QUALITY CONTROL OF COFFEE SUBSTITUTES: OCHRATOXIN A RESIDUES

Tiago Daniel Monteiro Vieira

Master in Quality Control

Faculty of Pharmacy of University of Porto

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Controlo de qualidade de sucedâneos de café: resíduos de ocratoxina A

Tiago Daniel Monteiro Vieira

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“Aos meus pais.”

Abstract

Contamination of food and feed with mycotoxins is a global health problem. Mycotoxins are fungal secondary metabolites that have adverse effects on humans and animals, resulting in illnesses and economic losses. Although not exclusive to coffee, mycotoxins are highly prevalent in this product, particularly Ochratoxin A (OTA). Alternative products such as coffee substitutes can contain coffee in various percentages plus roasted cereals, being both important sources of OTA contamination.

The aim of this work was to determine the levels of OTA in samples of coffee substitutes (mixtures with and without coffee, barley and chicory), commercialized in Portugal. The analytical method selected uses a solvent extraction with polyethylene glycol followed by OTA isolation and sample clean-up through immunoaffinity columns. OTA amounts were determined by reversed phase high performance liquid chromatography with fluorescence detection (HPLC-FLD). The method was validated in terms of linearity, precision and accuracy. Confirmation of OTA positive samples was achieved by gas chromatography with mass spectrometry.

In a total of forty samples analysed, including ten of soluble coffee, thirty-seven samples (83%) had OTA levels ranging from 0.05 to 5.76 µg/kg. Coffee samples had significantly higher amounts of OTA (p<0.05), followed by mixtures with and without coffee and later by plain cereal mixtures. A significant linear correlation between OTA amounts and coffee percentage in the mixtures was verified (p<0.01). The daily intake of OTA, estimated by the regular consumption of coffee and substitutes, indicates that these beverages are not an important source of OTA in the diet of the Portuguese population. Based on the maximum consumption of 4 beverages, prepared with 2 g of soluble powder each, soluble coffee contributes with an estimated maximum of 4.5 % (1.0% on average) of the provisional tolerable daily intake (PTDI), while the contribution from substitute with coffee, is about half compared to the previous one. Cereals contribution is extremely low, achieving an estimated maximum of 0.5% of PTDI.

OTA amounts in coffee substitutes are generally low and within the regulated and safety limits but the high incidence of OTA contamination in these products should not be disregarded.

Keywords: *soluble coffee, coffee surrogates, coffee substitutes, OTA, HPLC-FLD*

Resumo

A contaminação de alimentos e rações por micotoxinas é um importante problema de saúde pública. As micotoxinas são metabolitos secundários de fungos com efeitos adversos nos seres humanos e animais, causando doença e perda económica. Apesar de não serem exclusivas do café, as micotoxinas são altamente prevalentes neste produto, particularmente a Ocratoxina A (OTA). Os substitutos do café constituem produtos alternativos, podendo conter café e cereais torrados, ambos importantes fontes de contaminação por OTA.

Este trabalho teve como objectivo a determinação do teor em OTA em amostras de sucedâneos de café (misturas com e sem café, cevada e chicória), comercializados em Portugal. A metodologia analítica utiliza uma extração com polietilenoglicol seguida de isolamento e purificação por colunas de imunoafinidade. Os teores em OTA foram determinados por cromatografia líquida de alta eficiência com detecção por fluorescência (HPLC-FLD). Os métodos foram validados em termos de linearidade, precisão e exactidão. A confirmação das amostras positivas OTA foi conseguida por cromatografia gasosa com espectrometria de massa.

Num total de quarenta amostras analisadas, incluindo dez de café solúvel, trinta e sete amostras (83%) continham níveis de OTA entre 0,05 e 5,76 µg/kg. As amostras de café solúvel apresentaram teores significativamente mais elevados (p<0,05), seguindo-se as misturas com café e depois as misturas de cereais. Comprovou-se que existe uma relação significativa e linear entre a concentração de OTA e a percentagem de café nas misturas (p<0,01). O cálculo da estimativa da ingestão diária de OTA decorrente do consumo regular de café e seus substitutos indica que estes não são uma importante fonte de OTA na dieta dos Portugueses. Com base no consumo máximo de quatro bebidas preparadas com 2 g de pó solúvel cada, o café solúvel contribui com um valor máximo estimado de 4,5% (1,0% em média) para o limite diário tolerável estabelecido, enquanto a contribuição dos sucedâneos com café é de cerca de metade desta. Quanto aos sucedâneos à base de cereais, a sua contribuição é extremamente baixa, atingindo um valor máximo estimado de 0,5%.

Os níveis de OTA em sucedâneos de café são geralmente baixos e estão dentro dos limites regulamentados e de segurança, mas a elevada incidência de contaminação destes produtos com OTA não deve ser desprezada.

Palavras-chave: *café solúvel, sucedâneos de café, OTA, HPLC-FLD*

Works developed during the preparation of this thesis:

Book Chapters

1. Vieira, T., Cunha, S., Casal, S. (2013). Micotoxins in coffee. In: Coffee in Health and Disease Prevention. Chapter 38: Edited by Victor R. Preedy. *Elsevier*. Amsterdam. (in-press)

2. Vieira, T., Cunha, S., Casal, S. (2013). Analysis of the mycotoxin ochratoxin A in coffee. In: Coffee in Health and Disease Prevention. Chapter 129: Edited by Victor R. Preedy. *Elsevier*. Amsterdam. (in-press)

Papers to be submitted in scientific journals

- Ochratoxin A residues in commercial instant coffee and coffee substitutes.

Objectives

Ochratoxin A (OTA) is an ubiquitous nephrotoxic and carcinogenic mycotoxin. Although usually present in residual (µg/kg=ppb) amounts, there are several food sources contributing to its daily intake, which are the cause for public health problems. From a quality control perspective, the presence of OTA in processed foods is indicative of inappropriate storage conditions, particularly high moisture levels in fungal contaminated food.

In the case of coffee and coffee substitutes, being subjected to a roasting process, there is a widespread perception that OTA content is very low, as this is known to be partially destroyed under heat. As to coffee, the increased sensitivity of the available analytical methodologies has allowed the verification that OTA contamination is extensive and it depends, among others, on the initial contamination level and roasting intensity.

In Portugal, the coffee substitutes market (based mostly on barley, chicory and rye) is well established but information on OTA levels is inexistent, though being fundamental to control these products.

In this sense, the specific objectives of this work are:

- Adapt and apply an analytical methodology described for soluble coffees in the determination of OTA residues in coffee substitutes;
- Provide information on the content of OTA in coffee substitutes consumed regularly by the Portuguese population;
- Correlate OTA content with type sample analysed, the amount of coffee present in coffee substitutes, and the brand;
- Estimate OTA daily ingestion from the regular consumption of coffee and substitutes.

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"Um Grande Obrigado a TODOS!"

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Abbreviations and Symbols

AOAC	Association of Official Analytical Chemists
BEN	Balkan endemic nephropathy
bw	body weight
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
GC	Gas chromatography
HPLC	High-Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
IAC	Immunoaffinity columns
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
ISO	International Standards Organization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of detection
LOQ	Limit of quantification
m/z	mass-to-charge ratio
max	maximum
MS	Mass spectrometry
MW	Molecular weight
NMR	Nuclear magnetic resonance
OAT	Organic Anion Transporter Proteins
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTα	Ochratoxin α

PBS	phosphate buffered saline
PEG	Polyethylene glycol
pKa	Ionization Constant
RP	Reverse Phase
PTDI	provisional tolerable daily intake
PTWI	provisional tolerable weekly intake
g	relative centrifugal force
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
SPE	Solid Phase Extraction
TLC	thin layer chromatography
UV-VIS	Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry
t	temperature
v/v/v	percentage of volume by volume by volume
λ	lambda, "wavelength"
μ	“Mean of the data”
min	minutes
Nº.	number

THEORETICAL PART

1. Introduction

1.1. Coffee

Coffee drinking has become part of the modern lifestyle being spread all over the world. Coffee holds second position among all beverages after water, being also the second most valuable traded commodity in the world after petroleum [1]. Coffee is not consumed for nutritional purposes but mainly for its stimulatory effects and sensory properties, followed by other social and economic factors. Recent epidemiological studies revealed that an intake of 2 to 4 cups/daily could be effective in the prevention of coronary heart diseases, type II diabetes, certain forms of cancer, Parkinson and Alzheimer's disease, while being also beneficial in our daily life by improving cognitive functioning, digestion, and an overall sense of wellbeing [2].

Coffee is also the world's most widely traded tropical commodity, accounting for exports estimated on US$ 15.4 billion in 2009/10 [1]. Currently, the total coffee sector employs around 26 million people in more than 50 producing countries [1]. Coffee beans undergo several processes before they become the well-known roasted coffee. Once ripe, coffee berries are picked, processed, and dried. The beans are then roasted to different degrees, undertaking several physical and chemical changes that influence the final flavor [3].

Coffee is commercially available to consumers as roasted beans, roasted and grounded beans, as soluble or "instant" powder extracts or, more recently, also as pre-packed beverages. The soluble coffee industry was initially developed as an alternative to reduce coffee losses in times of abundance, but presently it boosted with grow of ready-to-drink beverages and flavoured specialties like cappuccino, vanilla or chocolate [4]. Soluble coffee is also available as blended with coffee substitutes, among other different roasted plant products, like chicory or barley that resemble coffee beverage characteristics, at a smaller price and with reduced caffeine content. One of the advantages of soluble coffee and related beverages is their practical and clean use (dissolve instantly in hot water) compared to other ways of making coffee. Yet, it shows an inferior taste to freshly brewed coffee.

1.1.1. Botany

The coffee plant is a relatively small shrub, originally from Africa, grown only in tropical and subtropical regions. The word “coffee” derives from the Latin name of the genus to which it belongs. *Coffea* L. a member of the family Rubiaceae. This term is used as a general term for the fruits and seeds of plants of the genus but it also applies to processed coffee as roasted beans, ground coffee extracts (instant coffee) and coffee brews [5]. *Coffea arabica* L. and *Coffea robusta* Pierre are the two main species grown in the world, which provides approximately 60% and 40% of the world´s coffee production, respectively [1], and their seeds are processed in commercial coffee products by diverse ways.

Coffee beverages are prepared from arabica only or a mixture of arabica and robusta beans. In general, coffee made from arabica beans has superior quality and taste, selling for a higher price than robusta. However, robusta plants are more disease-resistant, grow well in severe climates, produces a cup featuring substantial body, a relatively harsh and earthy aroma, but it does not give rise to the quality (sensory properties) achieved with arabica coffees. Also, caffeine content is distinct in the two species, with robusta presenting almost double amounts than arabica coffees [5, 6].

1.1.2. World Production and Consumption

Brazil has been the world's largest coffee producer for the last 150 years [7], currently producing about a third of all coffee worldwide. In 2011, Brazil produced a total of 2.7 million tonnes, more than twice the amount of Vietnam, the second largest producer, followed by Indonesia and Colombia (Table 1). Arabica coffee seeds are mostly cultivated in Central America and South America, while African and Southeast Asia countries are the largest producers of robusta coffee. Brazil, leading producer of arabica, is also the second largest producer of robusta, after Indonesia [8]. Robusta coffee production has been increasing over the last years, reducing progressively arabica’s market share. Its lower price and higher soluble solids content are two important characteristics for their increasing acceptance, particularly by the soluble coffee industry.

Global coffee consumption almost doubled over the last 40 years, from 4.2 million tonnes in 1970 to 8.5 million tonnes in 2011, an increase of 91 per cent. Coffee producing

countries consume around one third of their production and the remaining two thirds are traded internationally, being USA the biggest importer [9].

Coffee *per capita* consumption varies widely from country to country (Table 2). The Nordic European countries are the heaviest coffee consumers, varying from 8.4 to 12.0 kg/year to around 3 kg in the United Kingdom and most of Eastern European countries. Brazil, although being the second largest consumer of coffee, has a *per capita* mean consumption of 5.6 kg, thus ranking 14th. The United States have an annual *per capita* consumption of just over 4 kg comparable with the 5 kg described in Europe.

Table 1. World production of coffee raw beans in 2011 [10].

Continent	Raw coffee (x 1000 t)	Country	Raw coffee (1000 t)	Market share
Africa	1.119	Brazil	2.700	32%
Americas	4.768	Vietnam	1.277	15%
Northern America	3	Indonesia	634	7%
Central America	1.061	Colombia	469	6%
South America		Ethiopia	371	4%
and Caribbean	3.703	Peru	332	4%
Asia	2.486	India	302	4%
Europe	-	Honduras	282	3%
Oceania	85	Guatemala	243	3%
		Mexico	237	3%
World[a]	**8.457**	**Top 10 producers**	**6.846**	**81%**

[a] Aggregate (may include official, semi-official or estimated data); t = tonnes.

Table 2. The top 20 countries in coffee consumption per capita (kg)[11].

Rank	Country	Value*	Rank	Country	Value
1	Finland	12.0	11	Austria	6.1
2	Norway	9.9	12	Italy	5.9
3	Iceland	9	13	Slovenia	5.8
4	Denmark	8.7	14	Brazil	5.6
5	Netherlands	8.4	15	Greece	5.5
6	Sweden	8.2	16	France	5.4
7	Switzerland	7.9	17	Cyprus	4.9
8	Belgium/Luxembourg	6.8	18	Spain	4.5
9	Germany	6.4	19	Portugal	4.3
10	Canada	6.5	20	United States	4.2

*Annual consumption of coffee in kg per capita (green bean equivalent); Data reflects average consumption, in the period of 2005 to 2008.

1.2. Coffee Substitutes

Coffee popularity is still increasing despite the fact that there are also reports of negative side effects from excessive consumption, in particular with respect to its major alkaloid. Caffeine is a central nervous system stimulator, which has some effects on the cardiovascular system, with a slight increase in blood pressure and heart-beat, and may interfere with oral contraceptives or postmenopausal hormones effectiveness. Thus is generally advisable not to exceed the consumption of 2 to 4 cups/day [2].

The necessity to avoid caffeine by some consumers, alongside with certain times in history when coffee was scarce and its price too high, gave rise to the development of alternatives beverages that tried to simulate coffee flavour and aroma. Coffee substitutes or surrogates are usually parts of roasted plants (e.g. roots; seeds) which are made into a product which provides a "coffee-like" brew, being sold as a plain coffee substitute or blended with declared amounts of coffee. They are commercially available in the form of roasted cereal or, more frequently, as soluble or "instant" powders labelled. Being cereal-based extracts, they are also a complementary source of nutrients (carbohydrates, fibre, minerals, etc.) and important bioactive substances [12], essential to the proper functioning of the body. Surrogates that do not contain coffee are naturally free from caffeine and can be consumed at any time of day, even by children. They are also more affordable than plain coffee but their flavours are not as rich and characteristic as that of coffee [13-16].

In Portugal, commercial coffee substitutes are based mostly on roasted cereals (barley, rye and their malts) and chicory.

1.2.1. Main Coffee Substitutes and Processing

The two main coffee substitutes are chicory and cereals. Common chicory, *Cichorium intybus,* is a perennial plant growing to a height of about three feet, bearing blue flowers having a long tap root, and foliage which is sometimes used as cattle food. Chicory composition highlights proteins, minerals (calcium, phosphorous, iron) vitamins (vitamin A, B1, B2, niacin and vitamin C) and fibre. Their high content in inulin (about 70% of dry matter), makes it an excellent source of this polysaccharide, with interest for food and pharmaceutical industries [17].

Chicory plant is cultivated generally for its root, which is cut into slices, dried, and then roasted similarly to coffee (Figure 1). The effect of roasting upon chicory is to drive off a large percentage of water, increasing the reducing sugars, degrading a large proportion of the bitter extractives and inulin, and forming dextrin and caramel as well as the characteristic chicory flavour.

Figure 1.Dried (A), roasted (B) and ground (C) chicory used in the preparation of chicory coffee [18].

Cereal substitutes contain almost all type of grains mainly wheat, rye, oats and buckwheat. They are prepared in two general ways: by roasting the grains or the mixtures of grains, with or without the addition of substances such as sugar, molasses, tannin, citric acid etc.; or by first making the floured grains into dough and then baking, grinding, and roasting. Prior to these treatments, the grains may be subjected to a variety of other treatments such as impregnation with various compounds, or germination (malting process).

Cereal roasting is performed to a final temperature of 180 – 200ºC during a certain period of time. The effect of roasting on these grains and other substitutes is also similar to coffee: the crude fibre, starches, and other carbohydrates are decomposed, with the production of a characteristic flavour and an aroma faintly suggesting coffee. The roasted materials are then milled and commercialized as such, or subjected to an aqueous extraction of soluble solids, with hot water at 100ºC. The clarified extract is then concentrated and spray-dried to yield a soluble powder product that is used in the preparation of soluble coffee substitutes (Figure 2) [13, 15].

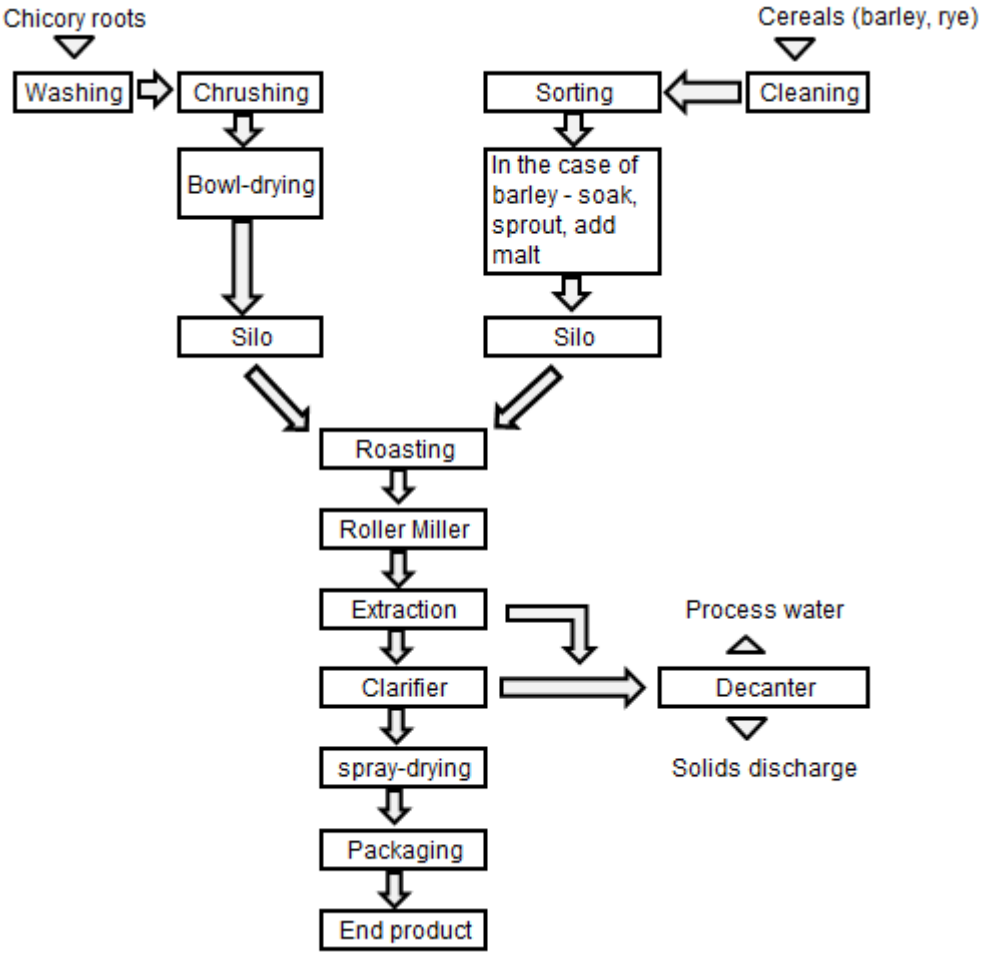


Figure 2. Production diagram for soluble coffee substitutes (based on [13, 15]).

1.2.2. Economic Importance of Soluble Coffee and Substitutes

Due mostly to its “practical” preparation and lower price, the soluble coffee industry has grown considerably in recent years. Still, the world trade of soluble coffee is equivalent to only 4 to 6% of that of green coffee beans. Exports of soluble coffee has grown from 216 thousand tons in 1990 to 270 thousand tons in 2000 and achieved an equivalent to 630 thousand tons of raw coffee in 2011 [19].

Europe is the main importer of green coffee and soluble coffee products (Table 3). Decaffeinated coffee and roasted coffee are the main exports of finished coffee products, given the strong development of coffee processing industry in Europe. USA is by far the largest non-EU destination (20.6%), followed by the Russia (12.4%), Ukraine (11.7%), and Switzerland (11.5%) [20].

Table 3. EU imports and exports of green coffee and products [20].

Coffee type	Imports (1000 t)			Exports (1000 t)		
	2008	**2009**	**2010**	**2008**	**2009**	**2010**
Green coffee	2.739	2.687	2.755	12	16	19
Green coffee decaffeinated	3	3	2	92	81	85
Roasted coffee	21	25	29	71	70	77
Roasted coffee decaffeinated	2	3	3	3	3	3
Soluble coffee*	44	41	44	39	39	39
Total	**2.809**	**2.759**	**2.833**	**217**	**209**	**223**

*Including roasted chicory and other coffee substitutes, their extracts and essences; t = tonnes

The five largest suppliers of soluble coffee products are coffee producing countries (Table 4). This is expectable because the preparation of soluble coffee can be regarded as a way to preserve coffee when its market offer is excessive or the prices low, adding value to the product. Until 2009, Brazil was unrivalled in the production of soluble coffee, followed by Ecuador. In 2010, Brazil lost its first position to Ecuador and Colombia took third place from Côte d'Ivoire, while India completed the top 5. Overall volume increased by 10.5% between 2008 and 2010.

Table 4. EU imports of soluble coffee from non-EU origins [20].

Country	Imports (1000 t)			%		
	2008	**2009**	**2010**	**2008**	**2009**	**2010**
Ecuador	10.44	10.18	11.55	25.2%	23.7%	24.3%
Brazil	12.03	11.12	10.08	29.1%	25.9%	21.2%
Colombia	3.97	3.52	3.68	9.6%	8.2%	7.7%
Côte d´Ivoire	5.17	4.22	2.77	12.5%	9.8%	5.8%
India	3.73	3.30	2.64	9.0%	7.7%	5.6%
Others	6.00	10.62	16.77	14.5%	24.7%	35.3%
Total External Trade	**41.34**	**42.97**	**47.48**	**100.0%**	**100.0%**	**100.0%**

Regarding EU exports of soluble coffee to non-EU destinations total volume remained unchanged (Table 5). In 2010 the three largest clients were the Russian Federation, Ukraine and Turkey. Exports to the United States dropped somewhat in 2010 after having increased in 2009 [20].

Table 5. EU exports of soluble coffee to non-EU members [20].

Country	Exports (1000 t)			%		
	2008	**2009**	**2010**	**2008**	**2009**	**2010**
Russian Federation	11.36	7.71	12.95	29.1%	19.7%	33.1%
Ukraine	8.66	8.95	9.56	22.2%	22.8%	24.4%
Turkey	1.81	2.13	2.33	4.6%	5.4%	5.9%
Switzerland	1.48	1.34	2.02	3.8%	3.4%	5.2%
United States	1.28	2.10	1.98	3.3%	5.4%	5.0%
Canada	1.34	1.25	1.11	3.4%	3.2%	2.8%
South Africa	0.67	0.57	0.87	1.7%	1.5%	2.2%
Israel	0.95	1.19	0.86	2.4%	3.0%	2.2%
Croatia	1.32	1.09	0.75	3.4%	2.8%	1.9%
Australia	0.76	0.74	0.70	2.0%	1.9%	1.8%
Others	9.42	12.16	6.04	24.1%	31.0%	15.4%
Total external trade	**39.05**	**39.23**	**39.16**	**100.0%**	**100.0%**	**100.0%**

t = tonnes

In comparison with soluble coffee, data on soluble coffee substitutes are scarce. It is possible to find production and consumption figures for cereals, but not on their food destinations. Still, the preparation of this type of beverage from roasted cereals represents a very small part in comparison with classical cereals usage (bread, pasta, etc.) or even cereals beverages (beer, whisky, etc.).

1.2.3. Coffee Market in Portugal

The average annual *per capita* consumption of coffee in Portugal is about 4 kg, which is one of the lowest consumption within Europe. Still, consumption has been growing over the years, with a 40 % increase since 1977 (Table 6).

Traditionally, coffee is consumed “out-of-home”, by way of beverages prepared on the moment from ground roasted beans, as the classical espresso or filter coffee which represent the largest market share (85.4%). Soluble coffee detains a small portion (14.6%) of the overall consumption of coffee. Still, over the last 14 years, total soluble coffee consumption has been growing, with a descending trend in out-of-home data [21]. Despite the stability of the market shares since 2008 (Table 6), both roasted and soluble coffee consumption have been increasing.

Table 6. Portugal coffee consumption: roasted and soluble coffee [21].

Year	Volume (1000 t)			Market share	
	Coffee Imports	Roasted coffee	Soluble coffee	Roasted coffee	Soluble coffee
1997	36.18	32.34	3.84	89.4%	10.6%
1998	41.76	37.08	4.68	88.8%	11.2%
1999	44.22	38.94	5.28	88.1%	11.9%
2000	38.76	33.96	4.86	87.6%	12.5%
2001	44.70	38.94	5.82	87.1%	13.0%
2002	43.20	37.32	5.88	86.4%	13.6%
2003	39.42	33.84	5.58	85.8%	14.2%
2004	41.10	35.04	6.06	85.3%	14.7%
2005	39.36	33.30	6.00	84.6%	15.2%
2006	40.26	33.90	6.36	84.2%	15.8%
2007	41.04	34.32	6.72	83.6%	16.4%
2008	39.24	32.46	6.84	82.7%	17.4%
2009	42.54	35.04	7.50	82.4%	17.6%
2010	45.00	37.08	7.92	82.4%	17.6%
2011	50.04	41.34	8.70	82.6%	17.4%
Average	**41.79**	**35.66**	**6.14**	**85.4%**	**14.6%**

The four biggest raw coffee suppliers are Vietnam (19.7%), Brazil (16.0%), Uganda (10.6%) and Cameroon (9.8%). The first two increased their volume and share in Portuguese imports. Spain is the main source of imported soluble coffee (68%), followed by the United Kingdom (20%). On the export side, Spain is also by far the highest export destination with 59% of the total volume, followed by Germany (9.4%) and Greece (9.1%)[20].

On the other hand, Portugal is the country in which the consumption of mixtures and substitutes of soluble coffee is more preponderant. The Portuguese "soluble coffee" market is characterized by a higher market share of mixtures (43.8%) with only 42.2% of plain soluble coffee (Figure 3) [22].

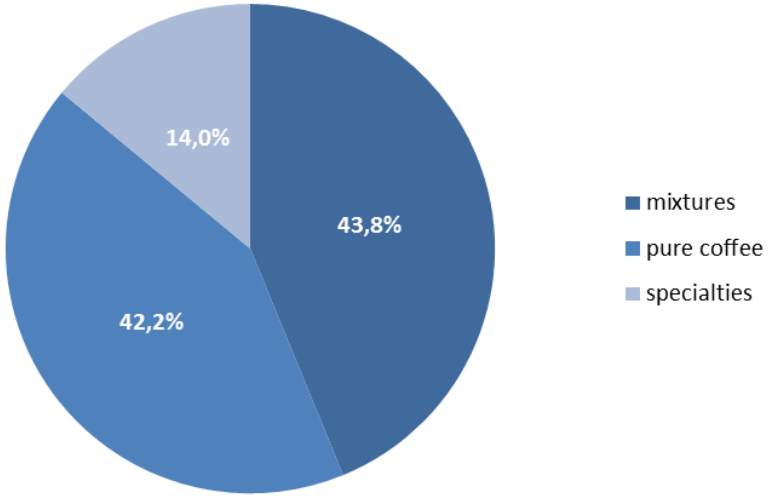


Figure 3. Characterization of Portugal market for soluble coffee and substitutes [22].

According to Food and Agriculture Organization of the United Nations (FAO) statistics, Portugal has been among the 20 highest trading countries (imports/exports) of coffee substitutes containing coffee over the last years [23]. In 2011, Portugal reached the 4th position in mass importations of this product, led by Slovakia, followed by Saudi Arabia and Spain. Imported quantities vary according to the needs that are not met by internal production. In 2011, there was a drop in imports of coffee substitutes containing coffee (Table 7), which may be in consonance with the increasing in exports between 2010 and 2011, resulting from an internal production growth.

Table 7. Portugal imports and exports of coffee substitutes containing coffee [23].

Trade quantity (tonnes)	2000	…	2008	2009	2010	2011
Imports	43	…	167	243	212	186
Exports	129	…	58	68	149	139

It is also interesting to denote that, despite its 12th position in 2011 regarding imported amounts, or 20th on exportation, Portugal ranks 4th in economic value on imports and 12th on exports, meaning that both imported and exported products have a higher value per mass in comparison with other countries. This could be an indirect assurance that both imported and exported soluble coffees are of recognized quality.

2. Mycotoxins in Coffee and Substitutes

Coffee quality, and hence their price, is determined by coffee species and varieties, geographic location, green processing method used and particularly the care given during coffee production. Mycotoxin contamination, however, represents an important safety issue regarding consumer´s health, creating trade barriers and significant economic losses in the producing countries. These compounds constitute, in general, highly toxic secondary metabolites produced by fungi, being mandatory to control traded products and, above all, to implement preventive strategies, particularly in the producing countries, in order to reduce this global health concern.

Contamination with mycotoxins is not exclusive to coffee, as 25% of the world agricultural crops are estimated to contain mycotoxins [24]. Cereals and derived products are regarded as the major human source of ochratoxin A (OTA) contamination worldwide, contributing with 50% of human daily intake of this mycotoxin [25]. In the particular case of coffee, contamination prevalence is increased, particularly for OTA, and in lesser extent for other mycotoxins, as aflatoxins and sterigmatocysitin. OTA is found in green coffee, roasted coffee and soluble coffee, being a key commodity in OTA research and regulation. Alternative products such as coffee substitutes may contain coffee in various percentages plus roasted cereals, being both important sources of OTA contamination.

The present chapter focuses on the description of OTA, the main mycotoxin associated with coffee and derivate products, its health effects, the legislation implemented, and the current data on coffee mycotoxin content. The issues associated with mycotoxin analysis in coffee will be detailed in a separate chapter.

2.1. Mycotoxins

Mycotoxins are low-molecular-weight toxic chemical compounds, of reduced volatility, formed as secondary metabolites by certain filamentous fungi that colonize crops, in the field or after harvest, capable of causing disease and death in humans and other animals through the ingestion of food products derived from these contaminated crops.

The term mycotoxin is derived from the Greek word "*mycos*" for fungus, and the Latin word "*toxicum*" for poison [26]. Nearly 400 types of mycotoxins have been identified so far but not all secondary metabolites synthesized by fungi "fit" within the group of mycotoxins [26]. Also, not all fungi are mycotoxigenic (mycotoxin producers) [27]. Six major classes of mycotoxins have been defined so far: aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxins, and ergot alkaloids [28]. They are produced by different fungi species and some can produce more than one mycotoxin. Mycotoxins are usually named after its parent fungal specie. The most important are those produced by fungus from *Aspergillus*, *Fusarium*, and *Penicillium* species, commonly associated with foods [29].

Globally, poisoning associated to the intake of contaminated food or feed, contact with mycotoxins by inhalation or skin absorption is designated as mycotoxicosis. Mycotoxin contact can result in acute or chronic toxicity on humans and animals (especially monogastrics) **[27]**. There is sufficient evidence from animal models and human epidemiological data **[30]** to conclude that mycotoxins are an important hazard to human and animal health, despite not being easy to establish a direct association between cancer or other chronic conditions with long term exposure to mycotoxins **[27]**. As detailed in Table 8, mycotoxins may cause deleterious effects on the central nervous system, on cardiovascular and respiratory systems, as well as digestive and urinary tracts, but the extensive list of effects includes also carcinogenic, mutagenic, teratogenic, and immunosuppressive ones. Indeed, immunosuppression is widely regarded as its most important effect, mainly in developing countries, by reducing the resistance to general infection **[26-28]**.

Mycotoxins are natural contaminants of agriculture commodities but appear to be restricted to certain environments and crops, with some being produced more readily than others, particularly in the moister seasons. Generally, mycotoxin presence is more common in parts of the world with deficient food handling and storage methods, with few regulations and guidelines to protect exposed populations **[27, 31]**. Mycotoxins can also be found in processed food from contaminated commodities, and their metabolic residues can be found in meat, eggs and milk from animals ingesting contaminated feeds, creating a secondary contamination route for humans **[29, 31]**.

Unfortunately, most people believe that simply by avoiding the use of visibly mould contaminated products the probability of acute mycotoxin levels is rare. However, being often invisible and tasteless, and relatively heat-stable within the range of conventional food processing conditions (80–121°C), their presence in food and feed represents a constant health risk for animals and humans **[31]**.

Table 8. Most relevant toxic effects from mycotoxins and associated mycotoxigenic fungi [28, 29, 32].

Mycotoxins	Major mycotoxin-producing fungi and main food commodities affected	Possible effects
Aflatoxins	*Aspergillus* sp. Cereals, oilseeds, dried fruits, **coffee**	Liver diseases (hepatotoxic, hepatocarcinogenic); carcinogenic and teratogenic effects; haemorrhages (intestinal tract, kidneys); reduced growth rate; reduction of performance; immune suppression.
Fumonisins	*Fusarium* sp. Maize	Pulmonary oedema; equine leukoencephalomalacia; nephron- and hepatotoxic; immune suppression.
Ochratoxins	*Penicillium* sp. Cereals, wine, beer, **coffee**	Nephrotoxic; carcinogenic; mild liver damage; enteritis; teratogenic effects; poor feed conversion; reduced growth rate; immune suppression.
Sterigmatocystin	*Aspergillus* sp. Cheese, **coffee**	Induction of hepatomas; pulmonary tumours; renal lesions; skin and hepatic tumours; diarrhoea.
Trichothecenes	*Fusarium* sp. Cereals	Digestive disorders (vomiting, diarrhoea, feed refusal); reduced weight gain; haemorrhages (stomach, heart, intestine, lung, bladder, kidney); oedema; oral lesions; dermatitis; blood disorders; infertility; degeneration of bone marrow; slow growth; immune suppression.
Zearalenone	*Fusarium* sp. Cereals	Estrogenic effects; vulvar oedema; vaginal prolapse; uterus enlargement; testicles and ovaries atrophy; enlargement of mammary glands; infertility; abortion.

2.2. Ochratoxin A (OTA)

OTA received its designation from *Aspergillus ochraceus*, where it was first isolated in 1965. Nowadays, several species of *Aspergillus* and *Penicillium* are known to produce OTA under certain conditions (Table 9), creating some difficulties in the identification of the fungi responsible for ochratoxin contamination [33]. OTA has been detected in a wide variety of agriculture commodities and livestock products. However, is the most frequently reported mycotoxin in coffee, in variable contamination levels, while references to other mycotoxins (aflatoxins and sterigmatocystin) are scarcer.

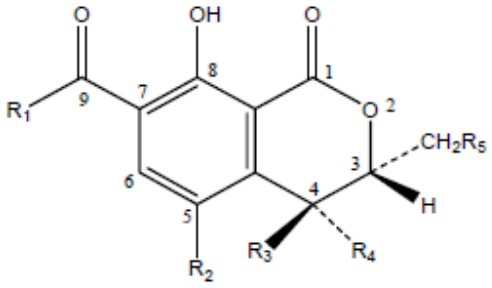
A. ochraceus fungi, has been predominantly associated with OTA contamination in coffee [34] while *P. verrucosum*, has been reported almost exclusively in cereal grain, especially wheat and barley [34, 35], important raw materials used in coffee substitutes manufacture. Generally in environments with lower temperatures, OTA is mainly produced by *Penicillium* species, while in warmer and humid areas (tropical and subtropical) is mainly produced by *Aspergillus* species [36, 37].

Table 9. Main characteristics for OTA-producing moulds development [37].

***Aspergillus* genus**	***Penicillium* genus**
Growth at higher temperatures:	
A. ochraceus 8-37ºC (Max. 31ºC); A_w up to 0.77	Growth at temp. <30ºC (Max. 20ºC)
A. carbinarius 32-35ºC; A_w up 0.82	A_w up to 0.8; pH between 6.0-7.0
A. niger 8-47ºC (Max.37ºC); A_w up to 0.72	Northern and central Europe and Canada
Warmer regions and the tropics	Contaminant of stored **cereal grain** and meat.
Contaminant of **coffee** raisins.	

Chemically, all ochratoxins (Figure 4) have a pentaketide derived from the dihydrocoumarins family coupled to β-phenylalanine through its carboxyl group in the 7-position [38]. So far, there are three recognized natural ochratoxins, designated as A, B and C. Figure 4 presents the general structure common to these different metabolites and shows the characteristic composition of each one. OTA is the most prevalent and relevant fungal toxin of this group [33], being chlorinated in R2 (Figure 5), while ochratoxin B, which is not chlorinated, and C (the ethyl ester of OTA) are less toxic and less common.

It is believed that the isocoumarin moiety is formed from acetate units via the pentaketide pathway carboxylated, and then chlorinated by chloroperoxidase to form ochratoxin α (OTα). The final step, linkage through the carboxyl group to phenylalanine, is catalysed by OTA synthetize. OTB is believed to be formed when chloride concentrations are low, and to some extent by dechlorination of OTA [33], Ochratoxin α (OTα) and ochratoxin β (OTβ) are products of hydrolysis of OTA and OTB, respectively, and are not toxic due to the absence of the phenylalanine moiety.



Common name	Abbreviation	R1	R2	R3	R4	R5
Ochratoxin A	OTA	Phenylalanine	Cl	H	H	H
Ochratoxin B	OTB	Phenylalanine	H	H	H	H
Ochratoxin C	OTC	Ethyl-ester, phenylalanine	Cl	H	H	H
Ochratoxin α	OTα	-OH	Cl	H	H	H
Ochratoxin β	OTβ	-OH	H	H	H	H

Figure 4. General structure common of ochratoxins [39]

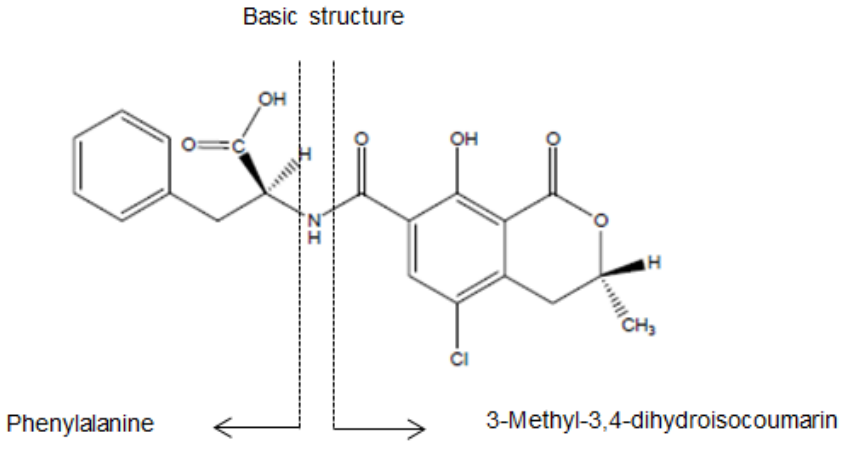


Figure 5. Chemical structure of ochratoxin A (OTA) [39].

OTA is a colourless crystal at room temperature under normal light, but exhibits a pH-dependent green or blue fluorescence with ultraviolet light, an important characteristic for its detection as will be detailed later on. Regarding OTA solubility, it is almost insoluble in water under acidic and neutral pH conditions but soluble in most organic solvents such as chloroform, ethanol, methanol, and xylene. OTA is light unstable, especially in very humid conditions, but is particularly stable in ethanolic solutions in the dark. As to thermal effects, OTA is stable under most food processing conditions, allowing the use of mild heat during its analytical determination. Only under extreme heating conditions some losses occur, as during coffee roast (> 220ºC), being therefore a safety barrier regarding coffee products.

The most important physical and chemical properties of OTA are detailed in Table 10.

Table 10. Physico-chemicals properties of OTA [38, 39].

pKa	7.1 (weak organic acid)	
MW	403.8 g/mol	
Structure	Crystalline structure, varying from colourless to white.	
Solubility	Acid and neutral pH – OTA is soluble in polar organic solvents (alcohols. ketones. chloroform), slightly soluble in water and insoluble in petroleum ethers and saturated hydrocarbons. Alkaline pH – soluble in aqueous sodium bicarbonate solution and in all alkaline solutions in general.	
Melting point	When crystallized from benzene, has a melting point of about 90ºC. When recrystallized from xylene, has a melting point of 169ºC.	
Stability	High stability: Resistance to acids and to medium temperature.	
Fluorescence	Acid medium – intense green fluorescence. Alkaline medium – blue fluorescence.	
UV-Vis	Ethanol 96% Absolute ethanol	λ_{max} = 213 nm (ε36.800 m^2/mol); λ_{max} =467 nm λ_{max} = 332 nm (ε6.400 m^2/mol); λ_{max} =428 nm
IR spectra characteristics	In chloroform	3380; 2988; 1723; 1674; 1528; 1425; 1381; 1304; 1260; 1170; 1140; 1107; 827 cm^{-1}
NMR H250- MHZ spectra characteristics	In deuterated chloroform	δ 12.70; δ 10.80; δ 8.55 (3H); δ 7.23; δ 7.15 (H aromatic); δ 4.71; δ 5.07 (CH); δ 2.78; δ 3.2 (CH_2); δ 1.55 (CH_3)
MS spectra characteristics	___	*m/z* 239/241 *m/z* 255/257 *m/z* 404

2.2.1. Exposure and Toxicity to OTA

Human exposure to OTA can occur by two routes: direct exposure via the consumption of contaminated foodstuffs, or indirect exposure through consumption of meat and other animal products, which contain trace amounts of mycotoxin ingested by animals (especially monogastrics) and their metabolites [35, 40]. Although, the main route is through food ingestion of contaminated food, mycotoxicosis can also be transmitted by the respiratory and dermal routes [27, 41].

The mycotoxicosis symptomatology depends on the type of mycotoxin consumed, amount and exposure period, animal species, breed, age, general health and immune status, and in lesser extent, synergistic effects involving genetic, nutritional status, and interaction with other toxics [27, 32]. Mycotoxicosis often remains unrecognized by health professionals, except when a large number of people are involved. Diagnose is difficult by the fact that moulds may be present without producing any toxin, and even when is detected, it's not easy to establish a direct relation given veterinary or human health problems [41]. Being metabolized in the liver, kidneys, and by microorganisms in the digestive tract, it is very hard to ascertain the chemical structure of its metabolites and the associated toxicity of the residues excreted or found in tissues [26].

OTA is a cumulative toxic with rapid absorption but has a slow elimination. The elimination half-life of this toxin is significantly longer in humans (35 days) and non-human primates (monkey, 34 days), than those observed in other mammalian species (mice, 40h; rat, 55-120h, and pig, 72-120h) therefore increasing its risk [42]. In humans and nonhumans primates (vervet monkeys), OTA is excreted mainly via the kidneys tubules using organic transporter proteins (OAT). However, OTA can be reabsorbed in all nephron segments, delaying elimination and thus increasing the risk of OTA accumulation in tissues [43], particularly in the kidneys, as previously mentioned. Being OTA elimination slower in humans than in all other species tested, higher toxicity is expected to occur [33]. Interesting studies regarding human exposure detected OTA in foetal serum, in amounts two times higher than maternal serum, indicating OTA passage through the placenta [44]. In addition, excretion to breast milk also appears to be relatively effective, as OTA has been identified in samples of human breast milk, thus constituting a threat to children in breastfeeding stage [45].

Nephropathy is the major toxic effect cause by OTA, being the kidney the main target organ. However, other effects such hepatotoxicity, neurotoxicity, teratogenicity, immunotoxicity and carcinogenesis (Table 11) are also common on several animals species, but its major toxicity location and extent depends on the sex, species and the cellular type of tested animals [42]. The genotoxic effect of OTA is still controversial, due to

contradictory results obtained in various microbial and mammalian tests. Still, after rat chronic exposure and pig sub-acute exposure, evidence of DNA-adducts formation was shown [46].

In humans, OTA is suspected of being the main etiological agent responsible for Balkan Endemic Nephropathy of (BEN) and associated urinary tract tumours [38]. BEN manifest as a chronic disease, untreatable and fatal, prevalent in rural areas of Serbia, Bosnia, Croatia, Bulgaria and Romania. BEN is characterized by marked anaemia, mild proteinuria, trivial urinary deposit, and in advance stage disease kidney size is considerably reduced as it its concentration capacity [43, 47]. Despite remaining unclear, OTA has been the prime suspect of this disease based on the similarity with swine nephropathy induced by OTA. The finding of DNA adducts in patients with BEN corroborate the correlation between the mycotoxin and nephropathy [33].

The mechanisms involved in the toxicity of OTA are still not completely understood. In order to explain the toxicity of OTA have been placed several hypotheses about the interaction of OTA and its metabolites. One of the main hypotheses appears to be related to protein synthesis inhibition. The phenylalanine group competes at the level of translation with the amino acid phenylalanine to the corresponding t-RNA, inhibiting phenylalanine transferase and then protein synthesis [48]. There are several studies suggesting the involvement of OTA capable of inducing oxidative stress mechanisms and formation of free radicals and reactive oxygen species, causing cytotoxicity [27]. This mechanism also interferes with mitochondrial membranes, being the suspect for the effects observed in mitochondria [33, 48]. The formation of various adducts in kidneys, liver and spleen was also observed in some mammal species [49]. This could interfere with the DNA repair systems and cell cycle controls systems and serve as an initiating point of carcinogenesis. The International Agency for Research on Cancer (IARC) classified OTA as a compound possibly carcinogenic to humans (Group 2B) [30].

OTA has been detected in a wide variety of agriculture commodities, livestock products, and processed food all over the world. Concentrations found in the final food products are generally lower than those found in raw materials since some processing steps can contribute actively to its reduction, such as malting, malt fermentation, bread production, coffee roasting, and the wine-making process.

To reduce exposure to OTA as much as possible, the Joint Expert Committee on Food Additives (JECFA) set a Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg bw/week [50], whereas the European Food Safety Authority (EFSA) Scientific Panel on Contaminants in the Food Chain had established a PTWI of 120 ng/kg bw/week[51].

Table 11. Effects of ochratoxin A on humans and animals [38, 43, 52-55].

Affected species	OTA Effects	Signs/Symptoms/Remarks
Humans	Nephrotoxic effects	Fatigue, headache, body weight loss, pale skin. Epigastric tension, respiratory distress and retrosternal burning. Kidney damage / dysfunction (endemic nephropathy): - degeneration of the proximal tubules; - kidneys reduced size and weight ; - diffuse cortical fibrosis (usually without signs of inflammation).
Swine	Nephrotoxic effects	Kidney damage / dysfunction (porcine nephropathy). Increased water consumption. Altered urine excretion (wet beds).
	Decreased performance	Reduced weight gain and increased mortality.
	Hepatotoxic effects	Liver damage.
	Genotoxic effects	DNA-adducts formation.
	Gastro-intestinal effects	Diarrhoea.
	Immunosuppression	Leucocyte count dramatically decreased. Necrosis and oedema in the lymph nodes, spleen and thymus. Decreased resistance to environmental and microbial stressors. Increased susceptibility to diseases.
Horses	Hepatotoxic effects	Liver damage.
	Hematopoietic effects	Haemorrhages and anaemia.
Poultry (turkey, chickens, broilers)	Immunosuppression	Lymphocytopenia and a regressed thymus. Increased susceptibility to diseases.
	Decreased performance	Retarded growth and decreased feed conversion. Reduced egg production, egg weight and weigh gain. Higher mortality rates.
	Residues	Residues present in liver, meat and eggs. Blood and meat spots in eggs.
	Nephrotoxic effects	Increased water consumption and renal dysfunction.
	Hepatotoxic effects	Liver damage.
Domestic animals (dog, cat and pet birds)	Nephrotoxic effects	Kidney damage.
	Gastro-intestinal effects	Vomiting, intestinal haemorrhage and dehydration.
	Neurotoxic effects	Anorexia. Tenesmus (inability to urinate/defecate).
	Decreased performance	Weight loss and postration.
	Immunosuppression	Tonsilitis (inflammation of the tonsils).
	Pathological changes	Epithelial degeneration - kidney. Mycohemorrhagic enteritis (cecum, colon, rectum). Necrosis of the lymphoid tissues.
Rodents (rats, mice)	Carcinogenic effects	Adenomas and carcinomas on renal tubular epithelium. Urinary tract tumours development. Mammary proliferative lesions (in female rates).
	Teratogenic effects	Fetal weights depressed. Largest number of malformations.
	Genotoxic effects	DNA- adducts formation (livers and kidneys of rats; livers, kidneys and spleens of mice).
	Neurotoxic effects	Damage in brain regions. Decreased of striatal dopamine and neural progenitor stem cells.

2.2.2. Regulation and Limits in Coffee and Substitutes

Human exposure to OTA led to the adoption of maximum levels for certain foods on several EU countries. In the particular case of coffee and derivate products, the presence of OTA is undesirable for public health protection and its effective control may be used as a trade barrier, affecting the economies of producing countries.

Various factors play a role in decision-making processes focused on setting limits for mycotoxins. These include scientific factors to assess risk, such as the availability of toxicological data, food occurrence data, and detailed knowledge about possible analytical methodologies. Socio-economic issues, such as commercial trade and food security, have also a major impact. Weighing these factors is important in the decision-making process to establish meaningful regulations and limits for mycotoxins in food and feed **[56]**.

According to Van Egmond *et. al.* (2007)[56], at least 99 countries had mycotoxin regulations for food and/or feed in 2003. In the European Union Commission Regulation (EC) Nº. 1881/2006 and its amendments, including No. 105/2010 [57, 58] for OTA, maximum limits were set for certain mycotoxins in different foodstuffs.

A recently conducted survey on the dietary exposure of OTA revealed that cereals and coffee are among the main sources of OTA intake in European populations **[59]**. The European Union has established maximum limits for OTA contamination for these food groups (Table 12).

Table 12. Maximum levels established among EU Member States as regards OTA in coffee and cereals [57].

Foodstuffs	Maximum levels (µg/kg)
Unprocessed cereals.	5.0
All products derived from unprocessed cereals, including **processed cereal products** and **cereals intended for direct human consumption**.	3.0
Roasted coffee beans and **ground roasted coffee**, excluding soluble coffee.	5.0
Soluble coffee (instant coffee).	10.0

Concerning all forms of coffee substitutes (with or without coffee), there are no regulatory standards establishing maximum limits for OTA in this specific product. A lack of legislation also exists to protect from OTA contamination in chicory.

It is totally prohibited to mix non-conform products with conform ones in order to reduce OTA contamination levels. There is also an interdiction to use chemical treatments for OTA decontamination in products for human consumption **[60]**, and so prevention must be the primary key point.

2.2.3. Incidence of OTA in coffee and substitutes samples worldwide

Selected surveys from all over the world have confirmed the presence of OTA in all forms of commercialized coffee (Table 13- 14).

Green coffee analyses (raw coffee) from all origins and both types of coffee (arabica, robusta) have shown that OTA contamination may be more frequent in some areas, but that no producing country is entirely free from contamination (Table 13). In coffee, the main contamination seems to occur during harvest of coffee cherries and processing of green beans, especially if it falls in the soil where prolonged contact favours fungal colonization and toxin production, depending on the weather conditions [61].

Table 13. Incidence of ochratoxin A (OTA) in commercial raw coffee worldwide.

Origin	Specie	Nº positive / Nº Samples	% positive	Range of OTA (µg/kg)	References
Africa	n.s	76/84	90%	0.5-48.0	[62]
Asia	n.s	11/18	61%	0.2-4.9	[62]
Brazil	n.s	2/3	67%	2.0-4.0	[63]
Central America	n.s	0/1	0%	<0.5[a]	[63]
Central America	arabica	0/6	0%	<0.1[a]	[64]
Columbia	n.s	3/5	60%	1.2-9.8	[63]
Ethiopia	arabica	0/1	0%	<0.1[a]	[64]
Indonesia	robusta	2/9	22%	0.2-1.0	[64]
Kenya	n.s	0/3	0%	<0.5[a]	[63]
Latin America	n.s	19/60	32%	0.1-7.7	[62]
New guinea	n.s	0/1	0%	<0.5[a]	[63]
South America	arabica	0/12	0%	<0.1[a]	[64]
Tanzania	arabica	5/9	55%	0.1-7.2	[64]
Thailand	robusta	50/50	100%	4.0-22.1	[65]
Unknown	robusta	2/3	67%	2.2-3.6	[63]
Unknown	n.s	1/1	100%	11.8	[63]
Yemen	arabica	7/10	70%	0.1-7.2	[64]
Zaire	n.s	1/1	100%	17.3	[63]

[a] detection limit of the method; n.s. – not specified.

As to roasted and instant coffees, it is easily perceived from Table 14 that the number of positive samples for OTA contamination is elevated, and the regulated limits for both roasted and instant coffee are sometimes surpassed (bold). Still, the majority of

samples are within the safety limits, supporting a prevalent but low contamination level. Still, these results should be interpreted with caution since some data are relatively old and might not give a real perception of the actual panorama. Also, diverse analytical methodologies were used to obtain these data, being therefore not directly comparable. Additionally, these data concern commercial samples, while particular attention should be given to low-grade coffee, incapable of achieving the standards imposed for exportation, and therefore being consumed in the producing country most likely without control for OTA.

Table 14. Incidence of ochratoxin A in commercial roasted and soluble coffee worldwide.

Coffee type	Retail country	Specie	Nº positive/ Nº Samples	% positive	Range of OTA (µg/kg)	References
Roasted coffee	Argentina	n.s.	13/24	54%	0.1-**5.8**	[66]
	Brazil	Arabica	23/34	68%	0.3-**6.5**	[67]
	Brazil	Arabica	41/47	87%	1.3–**31.5**	[68]
	Brazil	Robusta	16/16	100%	1.7–**23.3**	[68]
	Canada	n.s	42/71	59%	0.1-2.3	[69]
	Europe	n.s	?/484	-	<0.5[a]-**8.2**	[70]
	Germany	n.s.	273/490	56%	0.2-**12.1**	[71]
	Spain	n.s	35/72	49%	1.2-4.2	[72]
	Japan	n.s	5/68	7%	3.2-**17.0**	[73]
	Japan	n.s	3/23	13%	0.25-0.43	[74]
	Hungary	n.s.	22/38	58%	0.2-0.9	[75]
	United Kingdom	n.s.	17/20	85%	0.2-2.1	[76]
Soluble coffee	Australia	n.s	7/22	32%	0.2-4.0	[77]
	Argentina	n.n	17/22	77%	0.2-**13.6**	[66]
	Brazil	n.s	81/82	99%	<0.16[a]-10	[78]
	Brazil	Arabica	16/16	100%	0.5-5.1	[67]
	Canada	n.s	21/30	70%	0.1-3.1	[69]
	Europe	n.s	?/149	-	<0.5[a]-**27.2**	[70]
	Germany	n.s	5/9	56%	0.3-2.2	[77]
	Italy	n.s	46/48	96%	0.3-6.4	[79]
	Hungary	n.s	14/14	100%	0.5-6.5	[77]
	Japan	n.s	5/7	71%	0.6-1.1	[74]
	Russia	n.s	21/22	95%	0.2-3.5	[77]
	Salvador	n.s	6/6	100%	0.3-3.6	[77]
	Thailand	n.s	3/3	100%	1.3-1.9	[77]
	United States	n.s	3/6	50%	1.5-2.1	[77]

[a] detection limit of the method; n.s. – not specified.

Currently, there are insufficient worldwide data to support OTA occurrence in coffee substitutes. Being subjected to a roasting process similar to coffee, there may be a general perception that the OTA content is low. Nevertheless, the initial contamination levels from raw materials (coffee, chicory or cereals) might have a determinant contribution.

Only recently, an Italian survey [79] analysed some coffee substitutes samples containing coffee at different percentages (30-60%). The results showed (Table 15) a contamination by OTA on coffee substitutes, ranging between 0.33 and 0.52 µg/kg, which was below the limits set by the European Union for soluble coffee. Nevertheless, the incidence was very high. In fact, the toxin was present in all of the samples, demonstrating its thermostability at the high temperatures of the roasting process. However, given the limited number of analysed samples (*n*=5), it is difficult to understand the real incidence of OTA in this range of products and even if coffee was the major source this mycotoxin in the mixtures. There is also a lack of data relative to coffee substitute without coffee, of particular relevance when cereals are known to be the main source of OTA worldwide.

Table 15. OTA occurrence in coffee substitutes samples with coffee [79].

Type	OTA (µg/kg)[a]
Coffee:Barley (35:65)	0.52
Coffee:Chicory (60:40)	0.51
Coffee:Chicory (54:46)	0.51
Coffee:Barley (33:67)	0.38
Coffee:Barley (30:70)	0.33
Average	0.45

[a] Mean of triplicate samples; n=6

Therefore, the need of studying OTA contamination and exposure through coffee substitutes arises. Given the insufficient data on OTA amounts from coffee substitutes worldwide, and the lack of legislation for impose maximum limits of OTA levels in this matrix, despite its potential risk to human health. In countries like Portugal there is a huge variety of coffee substitutes in the market, which imposes itself the control of these products so as to be regulated in terms of consumption.

3. Analysis of the mycotoxin Ochratoxin A in Coffee and Substitutes

Being usually colourless and odourless, mycotoxins analytical detection and quantification in feed and foodstuffs is an important tool to prevent consumption of contaminated products, consequently safeguarding and protecting animal and human health. However, their vestigial amounts (ppb) require complex sample preparation steps and expedite equipment, not easily available on most quality control laboratories, reducing the capacity for in-house batch to batch control. Also, mycotoxins contamination within a specific food batch is not homogeneous, requiring adequate sampling rules for accurate results.

The analysis of trace compounds should be simple and fast to prevent spreading of dangerous compounds, and sufficiently economical to allow the development of monitoring programs for OTA in different matrices all over the world. However, only a few methods available in the literature include all the cited premises.

Until today, there are no methodologies developed to evaluate the content of OTA specifically in coffee substitutes. Thus, a literature review of analysis methods OTA can only be performed on the basis of matrix similarities, in this case soluble coffee. Indeed, in comparison with other food matrices, OTA analysis in coffee is further complicated by the presence of interfering coloured substances formed during roasting, such as melanoidins, with the ability to complex with OTA molecules [80]. Similar phenomena are expected to occur with the soluble extracts of roasted cereals and roasted chicory roots.

The present chapter will focus on OTA analysis in coffee products, particularly soluble coffee, being organized by the most common basic steps of extraction, clean-up, toxin analysis and confirmation (Figure 6), with a focus on the major advantages and limitations of each step. The usual amounts found in coffee products were already detailed in chapter 2.

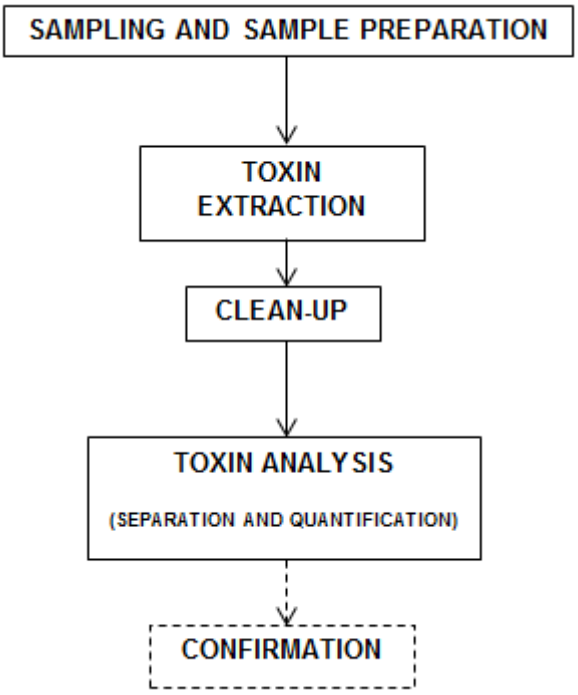


Figure 6. Diagram of general steps involved in OTA analysis.

3.1. Sampling and Sample Preparation

Sampling is an essential step in any monitoring activity regarding contaminants in food but, in the particular case of mycotoxins, it represents one of the most important steps for accurate results. Mycotoxins formation is associated to inadequate storage, thus its occurrence is sporadic and its distribution highly heterogeneous [81]. Therefore, inadequate sampling can be the main source of variance in the final test result for analysis of OTA, representing a major concern in terms of establishing average levels of OTA contamination in foods. Regulation (EC) No. 401/2006 [82], lays down the methods of sampling and analysis for the official control mycotoxins in foodstuffs, including coffee. Most sampling rules usually begin with a relatively large primary sample representing a lot, reducing it in bulk and particle size to a manageable quantity, and finally performing the analysis on a small representative portion. The basic act of mixing blends constitutes the “hot spots” of mould growth and subsequent OTA formation that can occur over a larger mass of whole grain reducing the distributional heterogeneity of OTA [81].

Sample preparation for analysis usually involves grinding and homogenization, or slurry preparation, before proceeding with OTA extraction. In particular, OTA concentration found in green coffee samples is known be dependent on the sample particle size, with higher contamination levels apparently found with smaller particular size (28 mesh versus 14 mesh) [83]. An adequate particle to the size of fine soluble coffee is determinant for an effective extraction, particularly in samples as hard as green coffee.

3.2. Extraction

All methods for the determination of mycotoxins require preliminary extraction of the mycotoxins from the food sample with a suitable solvent, before being possible to accurately quantify it by instrumental techniques.

For the determination of OTA in barley a mixture of $CHCl_3$ and H_3PO_4 was used as recommend by IUPAC/AOAC method [84]. The extract was further diluted with phosphate buffered saline (PBS) before purification with immunoaffinity columns [85]. In wheat, a number of extraction solvents are reported, including mixtures of toluene / HCl / $MgCl_2$ or $CHCl_3$ / ethanol / acetic acid and dichloromethane / H_3PO4 [85].

In coffee, OTA is usually extracted from green, roasted and soluble coffee with diverse organic solvents, water, or a mixture of both [63, 77]. Chlorinated solvents were initially used for OTA extraction in coffee samples [76] however, due to health concerns, these toxic solvents have been gradually replaced [86]. A proficiency study for OTA in roasted coffee samples employed a mixture of methanol with a 3% aqueous sodium bicarbonate solution (50:50) for extraction with consistent results [87]. It was also the selected extractive solvent of two collaborative studies conducted to evaluate AOAC official methods for both roasted and green coffee [88, 89]. Total aqueous extraction from roasted and instant coffee is also possible using 1% aqueous sodium bicarbonate or plain water [67, 86]. However, by testing different concentrations of $NaHCO_3$ a decrease in interfering compounds is observed with increasing $NaHCO_3$ concentrations up to 5%. Polyethylene glycol (PEG) was found to be useful in instant coffee samples [78], demonstrated to be effective (>80%) in the reduction of the effect of pigmented compounds that interfere in the process of antibody binding with OTA (in clean-up step) and thus co-extractive interferences on OTA retention time during chromatographic analysis.

3.3. Clean-up

The clean-up stage involves preliminary separation of the mycotoxin from other co-extracted interfering substances, being simultaneously an initial concentration step.

The use of conventional clean-up procedures such as liquid-liquid partition has been reported [90], as is or in combination with solid–phase extraction (SPE), in particular with celite column chromatography, being generally laborious and time-consuming [63, 91, 92]. However, it has been gradually replaced by commercially available and disposable SPE columns, namely those incorporating silica, C_{18} and immobilized antibodies for immunoaffinity chromatography [86]. By this way, reduction or removal of solvents in extraction processes and obtaining lower limits of detection and quantification has been achieved.

The introduction of immunoaffinity SPE sorbents as clean-up step for the analysis of OTA in coffee was considered a major advance in OTA cleaning techniques and, since then, has been applied to the development of new methods for OTA determination in several food. The immunosorbents, improving selectivity in the SPE step, have allowed the achievement of detection limits as low as 0.1-0.2 µg/kg **[77]**. In these immunoaffinity columns (IAC), liquid extract is forced through the column and OTA is bound to the activated antibody, interferences are removed with water and/or a saline solution (e.g. NaCl) and OTA is finally unbounded and eluted with an appropriate solvent, typically methanol, acidified or not. The immunological reaction in the SPE phase is specific for OTA, acting by a biomimetic approach, and therefore IAC columns represent a reliable tool for sample clean-up and allow direct processing of aqueous solutions, with benefits in terms of operational simplicity, speed and reduction of organic solvents use **[93]**. Also, methods using IAC can be easily automated, making possible a high throughput of samples per batch of analysis. Properly handled, IAC clean-up technique allows extracts of the highest purity to be obtained, which can be further separated either using normal or reversed-phase HPLC, thin-layer chromatography (TLC), gas chromatography (GC) and detected and quantified by visual and densitometric analysis, by fluorescence detection or mass spectrometry **[94]**.

However, IAC presents also some limitations, particularly associated with the generally low number of immobilized antibodies per SPE surface. Due to OTA high molecular weight (403.8 g/mol), that may lead to poor binding conditions, especially under sub-optimal conditions and in the presence of interferences **[83]**. Also, IAC tolerates only limited amounts of organic solvents. For optimal IAC performance, packing materials often

require specific environmental conditions such as aqueous media, well-controlled pH, ionic strength, and concentration levels of matrix components, which limit the optimization space for analytical method development. Another aspect is the susceptibility of the antibody-SPE materials to biodegradation, leading to short shelf life [93]. The elevated cost is also another disadvantage associated with IAC.

3.4. Chromatographic Analysis

The most widely used technique for analysis of OTA is liquid chromatography (LC), namely HPLC.

OTA natural fluorescence (Table 10 – Chapter 2) has been used for detection purposes, with several analytical methods using fluorescence detection published for the determination of OTA in coffee and achieving low detection limits (Table 13-15; Chapter 2). These methods include usually a previous purification step using either classical SPE or IAC. As to the chromatographic separation method, most HPLC methods use a reversed phase for separation and an acidic aqueous solution mixed with acetonitrile or methanol as mobile phase. Acidic mobile phases have been preferable for separation of OTA, because OTA is a polar compound, with a carboxyl group in the structure, and therefore it must be chromatograph in an ionised form to interact with the stationary phase [86].

Alternatively and more recently, detection by mass spectrometry (LC-MS) has been used, being one of the most advanced and powerful techniques available for the detection and identification of mycotoxins, particularly for those with reduced UV/VIS absorbance or native fluorescence. However, MS methods are time-consuming and require expert knowledge. Extraction and clean-up techniques still have to be applied prior to separation and detection in order to enable well-separated peaks without interference from matrix components [94].

Gas chromatography is also an alternative, with several methods published and, when associated with mass spectrometry, it allows a direct confirmation of the compound. Still, due to OTA's reduced volatility it requires derivatization as well as the previous described extraction and clean-up steps.

3.5. Confirmation

Apart from the accuracy and sensitivity achieved with the analytical methodologies, confirmation of OTA's identity is essential to ensure OTA analysis trueness.

OTA analysis by HPLC-FLD allows an ambiguous identification of its identity. Confirmation of OTA identity with this detection method is presumptively made by comparing the retention time of the chromatographic peak observed with a commercial standard. A simple way to confirm the identity of the metabolite is through the addition of a known amount of standard to the sample. If the detector response to increase in proportion to the amount of added standard is likely that the metabolite in question is the same as the default. Conversely, if two different chromatographic peaks appear, we are dealing with different compounds. However, the fluorescence properties of OTA may not provide a sufficient degree of certainty, considering the unequivocal analyte identification. To counteract this disadvantage, confirmation of the presence of OTA in various matrices is often achieved by methylation, with the consequence disappearance of the mother compounds and appearance of its methyl ester, with an increased retention time There are two distinct process of derivatization of OTA to the methyl ester: (1) the addition of 14% boron trifluoride methanol-complex (BF_3/MeOH) in methanol (80ºC) or (2) methanol and addition of diluted hydrochloric acid with reaction time overnight [95, 96]. After separation with HPLC and chromatographic analysis, the disappearance of the OTA peak and the appearance of a new peak (OTA methyl ester) at largest retention time confirmed OTA presence.

Only mass spectrometry, however, can provide a true confirmation of the compounds identity, by analyzing the molecular ion and fragments. This can be achieved with liquid or gas chromatographic systems using mass spectrometer detectors.

3.6. Reference Materials and Standard Methods for OTA analysis

Certified Reference Materials (CRMs) for various mycotoxins, including for OTA, are available in the "Joint Research Centre of the EU Institute for Reference Materials and Measurements" (http://irmm.jrc.ec.europa.eu/). The use of certified reference materials is very important, not only to ensure the accuracy of the methods, but also for the calibration of the equipment and to validate these same methods. This ensures control of the results obtained by a laboratory with a given analytical method. Certified reference materials are

relatively expensive and available in limited amounts. As such, it is advisable that the mycotoxin laboratories develop their own reference materials for routine use.

Standardisation of methods for determination of OTA in foodstuff is on-going, and several methods have been evaluated in interlaboratory studies. Available standard methods for cereals and coffee products and are listed in Table 16.

Table 16. Available standard references for OTA determination in cereals and roasted coffee.

Standard Reference	Title
EN ISO 15141-1:1998	Foodstuffs - Determination of ochratoxin A in cereals and **cereal products** - Part 1: High performance liquid chromatographic method with silica gel clean up (ISO 15141-1:1998)
EN ISO 15141-2:1998	Foodstuffs - Determination of ochratoxin A in cereals and **cereal products** - Part 2: High performance liquid chromatographic method with bicarbonate clean up (ISO 15141-2:1998)
EN 14132:2009	Foodstuffs - Determination of ochratoxin A in **barley** and **roasted coffee** - HPLC method with immunoaffinity column clean-up
EN 15835:2010	Foodstuffs - Determination of ochratoxin A in **cereal based foods** for infants and young children - HPLC method with immunoaffinity column cleanup and fluorescence detection

EXPERIMENTAL PART

4. Materials and Methods

4.1. Standard and Reagents

Ochratoxin A standard was purchased from Sigma (St. Louis, MO, USA). After complete dissolution in toluene-acetic acid (99:1), the exact concentration of the stock standard solution prepared was determined at 333 nm, after dilution, using 1 cm quartz cells, based on OTA molar absorptivity (ε = 5440 m^2/mol) described in literature (AOAC official Method 2001.1) [97]. This solution is known to be stable at -18ºC for at least 4 years [97]. Working solutions were prepared by appropriate dilution of the OTA stock solution in toluene-acetic acid (99:1). The solutions were stored at -18ºC, protected from light. The internal standards tested were of diverse purity and suppliers, and included phenylalanine, phenylalanine methyl ester, acetaminophen and ibuprofen, chosen due to its partial structural similarity with OTA. Due to OTA's toxicity, suitable protective measures were applied in all steps involving direct contact with OTA standards, namely protective gloves and manipulation in ventilated cabinet hoods. Also, all residues containing OTA were collected separately.

In the HPLC system, a mixture of 3.33% acetic acid, acetonitrile and methanol (30:35:35 v/v/v) was used for elution. HPLC-grade methanol and acetonitrile and glacial acetic acid (100%, EMSURE® ACS) were all acquired from Merck (Germany). Ultrapure water, purified with a "Seral" system (SeralPur Pro 90 CN), was used in the preparation of working solutions and mobile phase. The eluent mixture was degassed with a vacuum system. Toluene used to prepare the analytical standards was Pestanal® grade from Riedel-de Haën (Germany). For sample extraction two solutions were prepared: diluting solution, with 1% PEG 8000 and 5% sodium hydrogen carbonate, and a washing solution, prepared with 2.5% sodium chloride and 0.5% sodium hydrogen carbonate, both adjusted to pH 8.1. All the other reagents were of analytical grade, purchased from diverse suppliers.

In the identification of OTA, two derivatization reagents were used: boron trifluoride-methanol complex (14% solution in methanol) and *bis*-trimethylsilyltrifluoroacetamide (BSTFA), both from Sigma (St. Louis, MO, USA).

For confirmation of the analytical method effectiveness, a certified reference material was used. In the absence of a soluble coffee sample, or even a ground coffee one, a reference maize sample was acquired from TRILOGY® (USA), reporting to have 4.5 ± 0.9 µg/kg. This sample was preserved below -18ºC, until analysis.

4.2. Materials and Equipments

Diverse standard laboratory equipment's were used during sample preparation and extraction, including an analytical balance Metter Toledo AG204, a vortex mixer from Thermolyne Maxi Mix Plus ™, two centrifuges (Labofuge Ae, Heraeus Sepatech and Heraeus Sepatech Biofuge Pico, from Germany), a spectrophotometer SpectroStar nano (BMG Labtech, Germany), a pH measuring system pH-meter BASIC 20+ (Crison, Spain), a furnace (WTC Binder, Germany), among others.

For OTA isolation and concentration, immunoaffinity columns Ochraprep ® (R-Biopharm Rhone Ltd, Glasgow, UK) were purchased from Ambifood (Portugal). These columns contain an anti-OTA antibody, immobilized onto a gel material, and are filled with PBS for preservation. According to the manufacturer, they can be stored for 1 year under refrigeration (4ºC) or for 1 month at room temperature (below 30ºC) without performance loss. These columns were adapted to a solid phase extractor Visiprep ™ Supelco ® (USA), with a vacuum system, connected to a second empty large volume column to receive the sample extract (Figure 7).

Figure 7.Ochraprep® column coupled to an empty reservoir in a Visiprep system from Supelco.

Whenever possible, disposable material was used to avoid cross-contamination between samples. For reused material, and in accordance with the instructions given in the Ochraprep brochure, all material was decontaminated by 30 minutes soaking with solution of sodium hypochlorite (approx. 5%) followed by acetone (5% volume) for 30 minutes, and then fully rinsed with deionized water.

The determination of OTA was carried out in a HPLC system equipped with a quaternary pump (PU-1580) with in-line degasser system (Jasco, Japan), coupled to a manual injector (Rheodyne, USA) with a 20 µL loop and latter an AS-950 autosampler (Jasco, Japan). The detection system used was a fluorescence detector (FP-2020 Plus) also from Jasco (Japan). A reversed-phase C_{18} column (150 x 4.6 mm; 5 µm particle size, 110 Å pore size) (Gemini, Phenomenex, USA) was used for OTA chromatographic separation, preceded by a C_{18} guard column, also from Phenomenex (Figure 8). Collection and processing of chromatographic data was performed with Borwin ^{TM} PDA Controller Software 1.50 (JMBS, France).

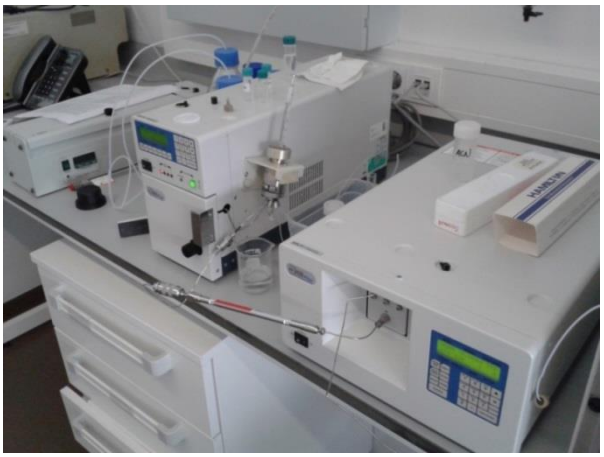


Figure 8.The HPLC system used in OTA separation (RP-HPLC-FD).

For OTA confirmation, a gas chromatograph 6890N interface to a 5975 Mass Selective Detector (Agilent, USA) (Figure 21) was used with a DB-5MS column (15 m x 0.25 mm x 0.25 µm film thickness, JW, USA) (Figure 9). The GC oven program was initially set at 150ºC and increased at a rate of 8ºC/min to 220ºC. After 5 min, the temperature is increased at a rate of 25ºC/min for 5 min to a maximum baking temperature of 290ºC. Helium was the carrier gas with a constant flow of 1 ml/min. The injection was made in splitless mode at 260ºC. The MS transfer line temperature was held at 280°C. Mass spectrometric parameters were set as follows: electron impact ionization with 70 eV energy; ion source temperature, 230ºC; MS quadrupole temperature, 150ºC and solvent delay 2 min. Selective ion monitoring was performed at ions 528, 529, 530, 531, 532, 604, 606 and 619.

Figure 9. The GC-MS system used in OTA identification.

4.3. Sampling

A total of 40 samples, among soluble coffee and coffee substitutes were collected from various commercial areas. Coffee substitutes contained barley, the most prevalent cereal, followed by barley malt, rye, and chicory, either with or without coffee in the mixture. Sampling included classical brands, as well as unbranded samples, also with important market representativeness.

All samples were identified and categorized based on the amount of coffee and / or substitutes declared in the ingredients list. The percentage of each constituent, when declared, was also taken into account. However, only coffee amounts are of mandatory labelling and some manufacturers do not indicate the amounts of each substitute. Based on this information, samples were grouped into five groups (Tables 18):

- coffee (n=10),
- mixtures of cereals with coffee (n=13),
- mixtures of cereals without coffee (n=16),
- barley (n=5) and
- chicory (n=1).

After acquisition and identification, samples were stored in their original packaging, at ambient temperature and protected from light.

Table 17. Identification and labelled composition of soluble coffees and substitutes.

Sample	Brand	Labelled composition (%)				
		Barley	Chicory	Malt	Rye	Coffee
Soluble coffee						
104	A	-	-	-	-	100
106	A	-	-	-	-	100
101	B*	-	-	-	-	100
102	A	-	-	-	-	100
143	C*	-	-	-	-	100
107	D	-	-	-	-	100
108	E*	-	-	-	-	100
109	B*	-	-	-	-	100
105	F*	-	-	-	-	100
103[a]	A	-	-	-	-	100
Mixtures with coffee						
114	E*	-	80	-	-	20
149	G	-	80	-	-	20
132	H	-	60	-	-	38
148	H	-	60	-	-	38
147	H	-	60	-	-	38
117	I	-	60	-	-	40
115	I	48	-	32		20
119	H	X	X	-	-	20
120	H	X	X	-	-	20
118	J	55	25	-	-	20
116	F*	55	25	-	-	20
138	K*	55	25	-	-	20
140	C*	55	25	-	-	20
Mixtures without coffee						
124	H	X	X	-	X	-
123	H	X	X	-	X	-
127	I	50	20	30	-	-
134	L*	50	20	30	-	-
129	D	X	X	X	X	-
146	M	X	X	X	X	-
126	H	X	X	X	X	-
130	N*	35	25	35	5	-
122	F*	35	25	35	5	-
137	K*	35	25	35	5	-
139	C*	35	25	35	5	-
Barley						
125	H	100	-	-	-	-
128	I	100	-	-	-	-
121	F*	100	-	-	-	-
135	L*	100	-	-	-	-
136	K*	100	-	-	-	-
Chicory						
131	O	-	100	-	-	-

(*) unbranded; X: unknown percentage content ; ([a]) 65% roasted coffee and 35% green coffee;

4.4. Analytical Methodologies for OTA Analysis

The method selected for extraction and quantification of OTA in soluble coffee and soluble coffee substitutes/cereals a reversed-phase liquid chromatography with fluorescence detection preceded by extraction and clean-up by immunoaffinity, was based on a procedure described by Almeida *et al*. (2007)[78]. The method was originally developed for quantification of OTA in wine, grape juice and beer samples, being also adapted by the authors for use in soluble coffee samples. In the present work, the method suffered some adjustments in order to improve its efficiency and adapt to coffee substitutes samples.

In summary, the principle of the extractive method used is based on a solid/liquid extraction with a solution containing PEG 8000 under alkaline media ($NaHCO_3$), followed by centrifugation and filtration to remove insoluble sample components, and cleaned-up/concentration on an immunoaffinity column. OTA is eluted from the IAC column with methanol, taken to dryness under a gentle nitrogen stream, re-dissolved in the HPLC eluent and quantified by reversed-phase liquid chromatography with fluorescence detection, making use of OTA natural fluorescence.

The process layout can be easily followed in Figure 10. For an easier explanation of the techniques below, each step was identified with capital letters.

4.4.1. Sample preparation

Prior weighing samples for analysis, the content of each sample flask was mixed to ensure sample homogeneity (**A**). Then, an accurate amount of sample mass around 2.5 g of homogenised sample was weighed into 50 mL conical Falcon tubes (**B**).

4.4.2. Extraction

To the weighed samples, 12.5 mL of water (**C**) plus 12.5 mL of diluting solution (**D**) were added, followed by vigorously vortex-mixing in for 5 minutes (**E**). Samples were left to macerate for 5 minutes (**F**) and centrifuged 5 min at 16,060 x g (**G**). The supernant was collected to a second centrifuge tube (**H**) and the remaining sample residue was re-extracted again with further 12.5 mL of water and 12.5mL of diluting solution (**I**). The supernatants were combined (**J**), and filtered through Whatman No.4 filter paper (**K**).

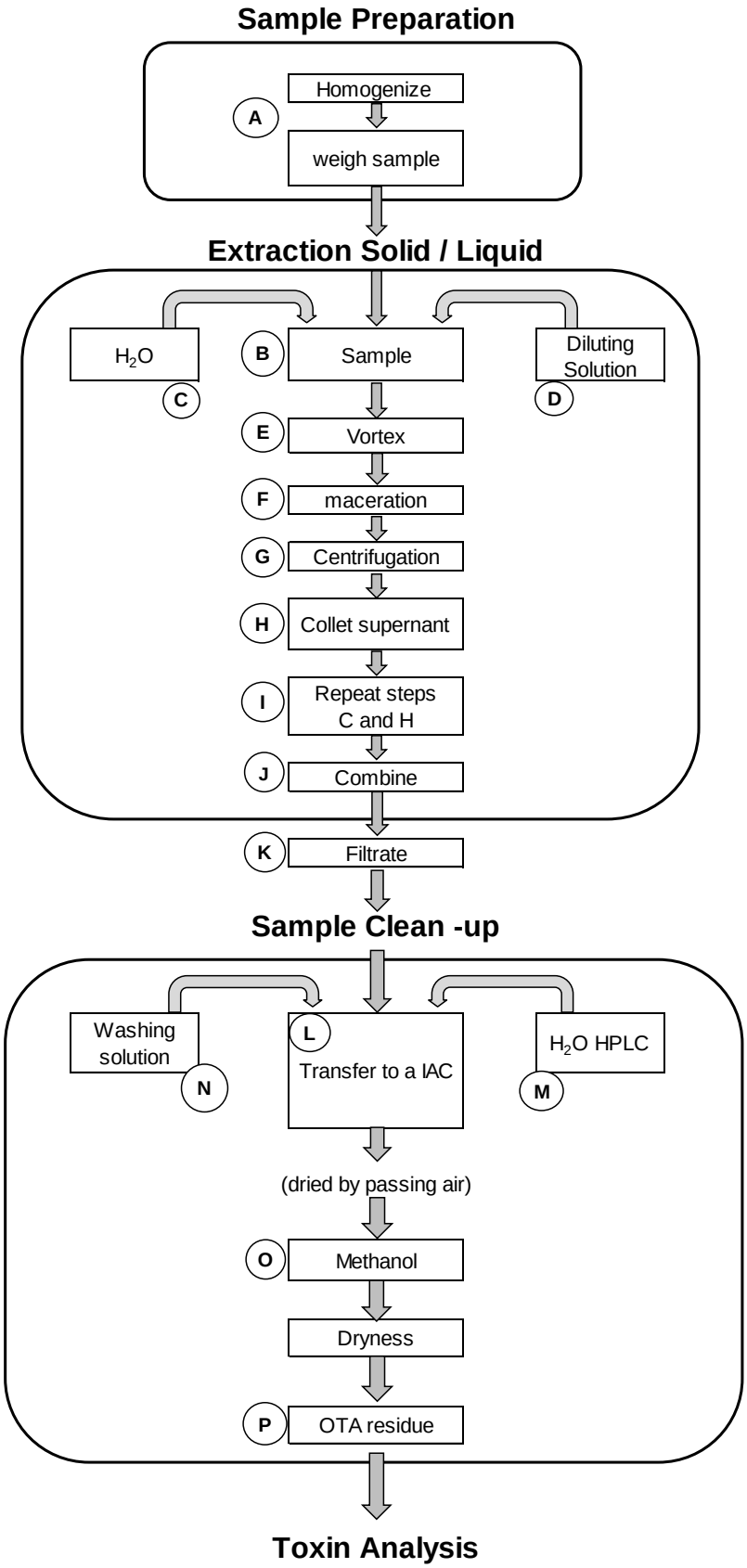


Figure 10. Resume of OTA extraction methodology.

4.4.3. Sample clean-up / concentration

After adapting a reservoir to the immunoafinity Ochraprep column, 40 mL of the sample extract (**K**) were passed slowly through the column (**L**). Under this stage, OTA is adsorbed into the column binding sites, being essential to use an adequate flow and avoid exceeding OTA binding capacity. Unbounded material was removed with 10 mL of the washing solution (**M**), followed by 10 mL of water (**N**) and by air passage to remove residual liquids. Finally, OTA is slowly eluted from the column with 2 mL of methanol (**O**), with back-flushing aid as recommended by the manufacturer. Then elute is taken to dryness under a gentle stream of nitrogen. The residue was dissolved in 300 µL HPLC mobile phase before chromatographic analysis (**P**), being 20 µL injected in the HPLC system.

4.4.4. Chromatographic Analysis

Each sample extract was injected in triplicate, at ambient temperature (about 22ºC) at an eluent flow rate of 1.0 mL/min. The fluorescence detector was operated at an excitation wavelength of 333 nm and emission wavelength of 476 nm, gain 100.

For quantitative determination of OTA based on the chromatographic peak areas, standard solutions were previously injected. For this purpose, appropriate volumes of OTA working solutions, obtained by serial dilutions, were taken and analysed in the HPLC system. After identification of the OTA peak and confirmation of chromatographic linearity, the process was repeated with standards subjected to the entire analytical protocol: diluted with 25 mL of water and 25 mL of dilution solution and 40 mL were taken and submitted to the immunoafinity adsorption and elution, being taken as samples.

For sample quantification and after determining the OTA mass by the calibration curve, the amount of OTA in the samples (C_{OTA}; in µg/kg) were calculated from the following equation (Equation 1):

$$C_{OTA} = M_{OTA} \times \frac{V1}{V2} \times M\ sample \quad (1)$$

Where M_{OTA} is the mass of OTA (in µg) in the volume solution of the dried elute when dissolved (300 µL) before injection on column, determined from the calibration graph; V_1 is the extractive volume (50 mL); V_2 is the volume solution taken for analysis, prior collected from the filtrate to clean-up (40 mL) and M is the sample weight.

4.4.5. OTA confirmation

Despite the high specificity of the immunoaffinity column, and the presence of a chromatographic peak in the samples at the exactly same retention time of OTA standard, the trueness of the peak was tentatively confirmed by methylation, based on a procedure described by Pittet *et al*. (1996)[77]. The availability of a gas-chromatographic system with mass detection made a second confirmation possible, based on a paper of Soleas *et al*. (2001)[98], after derivatization with BSTFA.

I – Derivatization with boron trifluoride

Two positive samples for OTA (#115 - coffee substitute with coffee and # 107 - soluble coffee) were tested. The remaining extract after HPLC analysis was taken to dryness under a stream of nitrogen at 40ºC, and re-dissolved in a 200 µL of BF_3/methanol complex (14% solution in methanol). The solution was mixed thoroughly and heated for 10 min. 80ºC, and allowed to cool to room temperature. Then 20 µL of derivatized extract was injected into the HPLC system under the same conditions as specified above.

II – Derivatization with ethyl acetate/ BSTFA

A second extract of the same positive samples selected in the first confirmation method tested (mentioned above), was brought to dryness under a gentle stream of nitrogen and derivatized with 100µL of ethyl acetate/ BSTFA (1:1) for 2h at 70ºC. A 1 µL aliquot of the derivatized extract was injected via a splitless injector in the gas-chromatographic system under the same conditions as specified above.

4.4.6. Statistical analysis

The statistical analysis of the results of different measurements was carried with computer programs Microsoft ® Excel 2010 (Microsoft Corporation, USA) and IBM SPSS Statistics 21.0 (IBM Corporation, Software Group, Somers, NY, USA).

5. Results and discussion

5.1. Optimization of the analytical methodology to quantify OTA

5.1.1. Optimization of chromatographic conditions

The chromatographic conditions reported by Almeida *et al*. (2012)[78] were tested. Despite the different chromatographic column, from a different brand and of smaller dimensions in our case (25 cm *vs* 15 cm), the eluent was the same, eluting in isocratic mode at 0.5 mL/min. Under these conditions, an adequate separation of OTA peaks from potentially interfering ones in sample extracts was verified. However, due to some instability in the HPLC pump, probably from the low viscosity of the eluent mixture, attempts to increase the solvent flow without resolution loss were made. Indeed, an increase to 1.0 mL/min allowed a better stabilization of the HPLC system without resolution loss, achieving a high precision of OTA peak retention time.

With the adjustments described, and using the fluorescence detector at gain 100, the chromatographic linearity was tested using OTA standards solution ranging from 0.2 to 300 ng/mL as proposed by Almeida *et al*. (Figure 11). Good linearity was achieved (r^2=0.999).

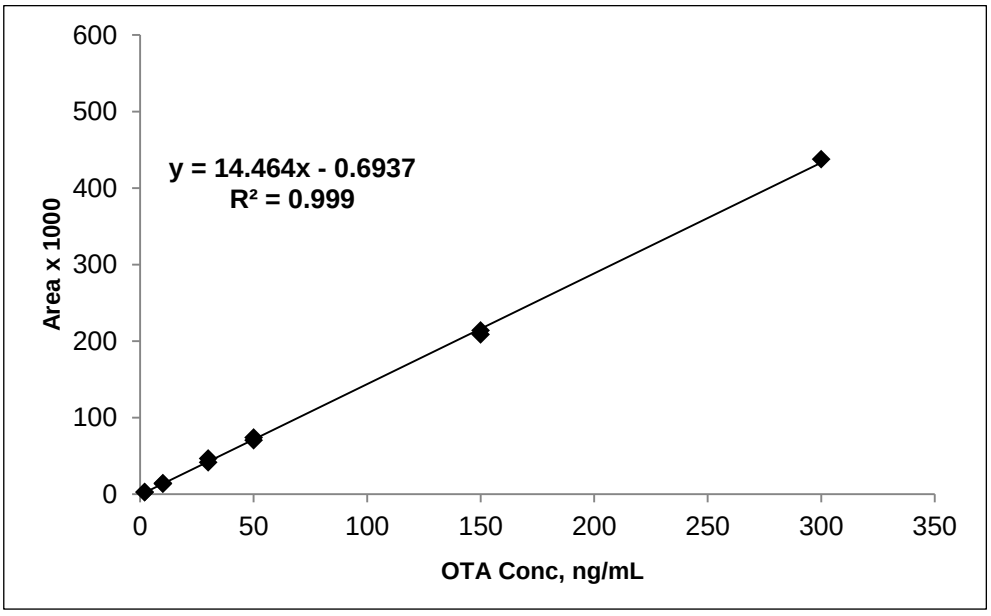


Figure 11. Linearity determined for OTA with direct standard solutions range of 2-300 ng/mL.

Several attempts to use an internal standard were made, as it would increase the precision of the method. However, the compounds tested, chosen to present a chemical similarity with OTA and fluorescence, co-eluted or eluted to closely. The internal standard would have been particularly useful to compensate for OTA loss during the extraction method or for volumes variability during the entire analytical method.

5.1.2. Optimization of extraction conditions

The methodology described by Almeida *et al*. (2012)[78] was applied by the authors to soluble coffee. However, when directly tested on coffee substitutes, several complications occurred. In particular, the amount of insoluble mater in the extractive solution was higher, reducing the efficiency of the filtration step and later imposing an increased and variable elution time of the sample extract through the Ochraprep column. Indeed, those samples containing higher cereals amounts were also the ones with higher amounts of insoluble particles. The richness of cereal extracts in insoluble fibre, particularly inulin in barley, could have a contribution in this process. In the coffee samples or when coffee was present in the mixtures, the granulometry of the dry samples extracts was generally higher, the residue was comparatively smaller and the filtration and elution steps occurred faster. Based on this observation, the extraction steps included a centrifugation before the filtration step, avoiding subsequent clogging. In order to increase the extraction efficiency in the presence of insoluble particles, a double extraction was performed to increase the extraction efficiency.

As an example, chromatograms corresponding to a sample extracted after the modifications performed in the present methodology are presented ahead (Figure 12). Clearly, OTA is identifiable in sample extract with a characteristic peak at RT 8.7 min after the optimization of the extraction method, by comparison with a standard solution (Figure 13), under same extractive conditions.

Aware that the extractive method, and particularly the imunoaffinity step, might impose some variability in OTA amounts, we have chosen to use calibration curves with standards submitted to the entire analytical protocol, as will be detailed under the validation chapter.

We have latter verified that Vechio *et al*. (2012)[79], in their article on soluble coffee, have also included the analysis of six coffee products with coffee and barley or chicory. The results achieved were previously discussed in the Theoretical part. The methodology used by this authors is similar to the one described by Almeida *et al*. (2012)[78], using Ochraprep column, except the extractive solution composition that has a different saline

composition. However, these authors have not attempted to change their method for soluble coffee, probably because on 100% cereal samples were analysed, our major source of analytical problems.

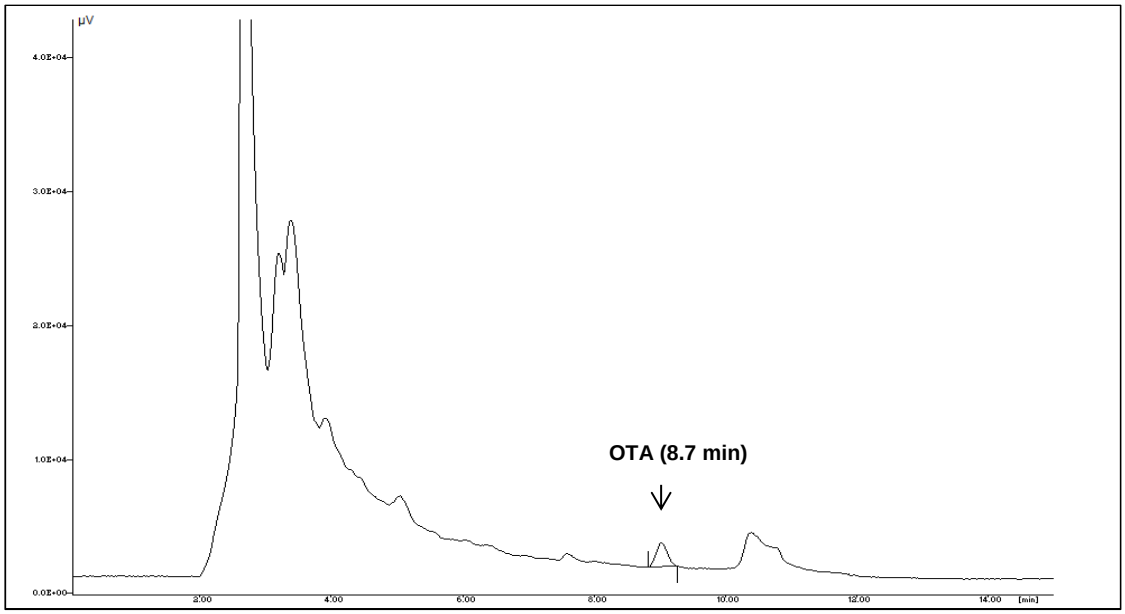


Figure 12. Chromatogram of a coffee substitute sample after optimization of extraction conditions.

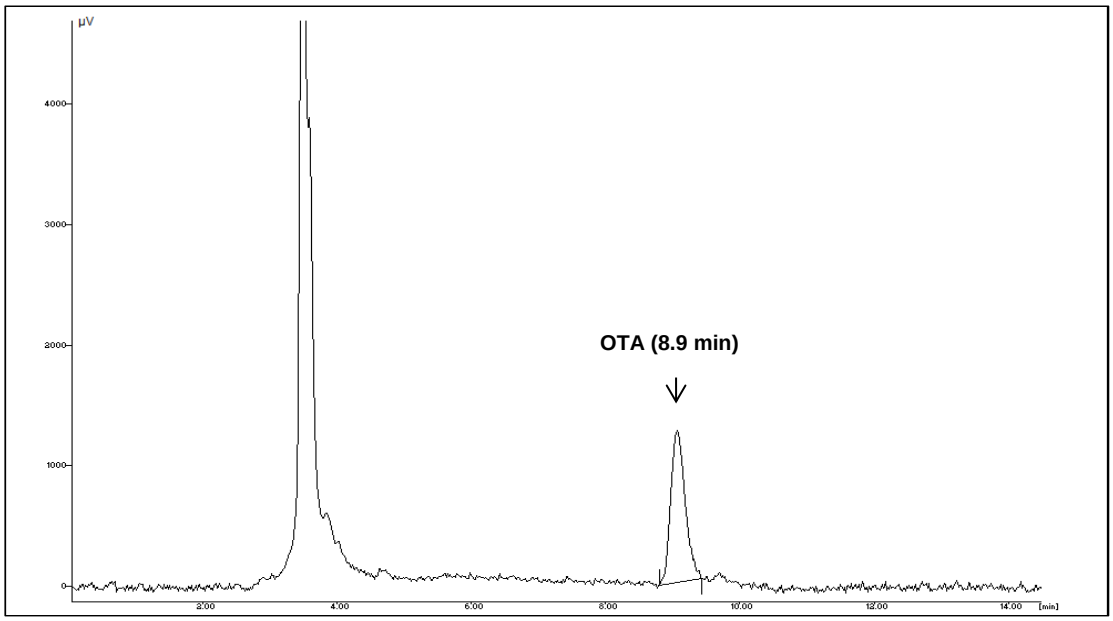


Figure 13. Chromatogram of an OTA standard solution (2 ng/mL).

5.2. Validation

The quality of a chemical method is sustained by its comparability, traceability and reliability. Each method must be validated at the time following its development, or whenever there is a change such as, for example, using the method for analysing different matrices. The analytical parameters usually required for methods validation include: linearity and working range, precision, accuracy, detection and quantification limits, important tools to ensure selectivity, specificity and robustness [99, 100].

Owing to the modifications made to the method originally described by Almeida *et al*. (2012)[78], imposed by the attempt to use the method to analyse coffee substitutes, a new validation is mandatory in order to attest if the results are reliable and suitable for quality control purposes. The parameters evaluated were: linearity and working range, sensitivity, precision (by coefficient of variation), accuracy (through reference sample), and limits of detection and quantification.

5.2.1. Linearity and working range

Linearity corresponds to the capacity of the analytical method to generate results proportional to the analyte concentration within the working range specific of the element in analysis [99, 100]. As mentioned earlier, standard solutions at different concentrations were prepared from successive dilutions of a stock solution of OTA, corresponding to approximately 0.1 to 12 µg/kg, based on the analytical protocol defined for samples, equivalent to a final concentration in the injected solution of 0.6 to 75 ng/mL. All standard amounts were diluted with 50 mL of water and diluting solutions and 40 mL were subjected to an IAC extraction, similarly to the samples protocol. After dryness of the methanolic extract and reconstitution in HPLC eluent, each standard was injected in triplicate.

The linearity study was performed by analysing the correlation coefficient of the calibration curve obtained by injection of standard solutions covering the values found in all samples. By this procedure the calibration curve includes other factors than the chromatographic ones, namely the reagents effect, their extraction and also the analyst errors. The results of the linearity verified in the working range tested are shown in Figure 14. The correlation coefficient had always a value greater than 0.99, which shows a reduced dispersion for results after the extractive protocol, and therefore low uncertainty of the estimated regression coefficients. Being performed after extraction, the correlation established compensates for the method variability and potentially some “matrix” effect derived from the analytical protocol.

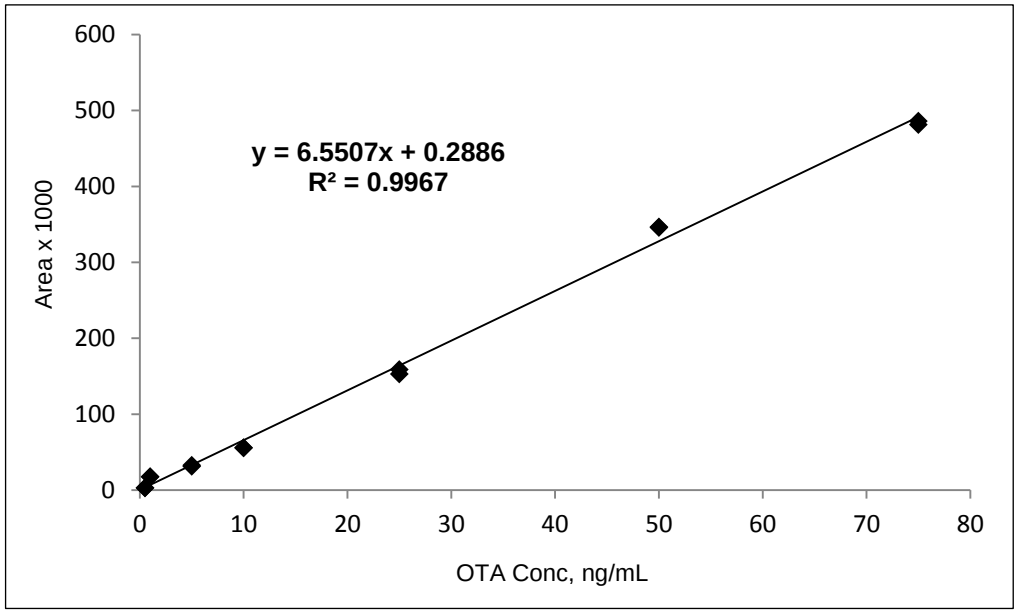


Figure 14. Calibration curve with extracted OTA standard solutions (n=3).

5.2.2. Instrumental precision and repeatability

The precision of the analytical process is a parameter that evaluates the proximity between various measurements made on the same sample [99, 100]. It includes the instrumental variability, in this case derived from the chromatography, and the extractive variability, involving all steps preceding the chromatography.

The instrumental precision was calculated as the relative standard deviation of the peak area obtained from three consecutive injections of sample extracts: two samples of coffee substitute (with and without coffee), one soluble coffee and a reference sample (maize). As it can be seen from the coefficients of variation indicated in Table 18, there is good instrumental precision associated with the method. The precision obtained with direct standard solution was even greater derived from the reduced baseline chromatographic noise (data not shown).

Table 18. Instrumental precision.

OTA positive sample	Mean Area	SD	RSD%
coffee substitute	17576	39	0.2%
coffee substitute with coffee	115843	124	0.1%
soluble coffee	254438	2731	1.1%
reference material (maize)	176420	767	0.4%

Repeatability was calculated based on the average content for the correlation with average area of the peaks corresponding to two simultaneous extraction of the same coffee substitute sample, each one injected in triplicate. Repeatability was also analysed with a sample of soluble coffee. In this way the results obtained presented low coefficients of variation (<5%), which indicates a good repeatability of the method (Table 19).

Table 19. Repeatability

OTA positive sample	Mean OTA (µg/kg)	SD	RSD (%)
coffee substitute with coffee	2.69	0.13	4.9%
soluble coffee	6.00	0.27	4.5%

5.2.3. Accuracy

Accuracy is defined as the correlation between the actual value of the analyte in the sample and those estimated by the analytical procedure. Since the objective was to verify the accuracy of the method applied in coffee substitutes samples, and in the absence of a commercial available reference sample for this matrix, a reference sample of maize with 4 ± 0.9 ppb OTA was used. The reference sample was subjected to the same extraction process defined for coffee substitute samples. Under our experimental conditions, the reference sample gave an OTA content of 3.3 ± 0.01 µg/kg, which is within the range defined by the manufacturer, and therefore validates the methodology.

5.2.4. Limits of detection and Quantification

The detection (LOD) and quantification (LOQ) limits were established as three and ten times, respectively, the standard deviation obtained from the analysis of the blank instant coffee extract (n = 10) at the retention time of OTA. The LOD and LOQ, correspond to the lowest concentration of analyte that can be detected or quantified, respectively, with acceptable accuracy and precision [99, 100]. Values obtained are shown in Table 20. The limits of detection and quantification obtained in the work of Almeida *et al.* (2012)[78] in instant coffee samples were, respectively, 0.16 µg/kg and 0.52 µg/kg respectively, slightly higher than those determined under our analytical conditions. Our values were more similar to those reported by Vechio *et al*. (2012)[79], also for soluble coffee, with 0.05 and 0.2 µg/kg, respectively. Thus, the technique used for OTA

determination with HPLC showed to be applicable for quantification of OTA content in instant coffee samples, given reliably analytical concentrations of OTA starting from 0.15 µg/kg.

Table 20. Limits of detection and quantification.

Sample	limit of detection (µg/kg)	limit of quantification (µg/kg)
blank instant coffee extract	0.05	0.15

Based on all the parameters used in the validation methodology used in this study, and in comparison with both Almeida *et al*. (2012)[78] and Vecchio *et al*. (2012)[79], it can be said that this technique presents equivalence in terms of precision, sensitivity and selectivity for coffee substitutes analysis. Thus, the analytical parameters needed to assess the reliability of the analytical results and to ensure the quality of the assay were obtained with success, thus allowing the confirmation of the performance of the method of determination of OTA in coffee substitutes by HPLC.

5.3. Confirmation of Ochratoxin A presence

The most frequent confirmation technique in positive samples in based on the method described by Pittet *et al*. (1996)[77], where OTA is methylated, with the consequent disappearance of the peak at OTA characteristic retention time and appearance of a new peak at higher retention time. Therefore, this was first attempted with two positive sample, one of plain coffee (#107) (data not shown) and one of a coffee mixture with cereals (#115). The identification of methylated OTA retention time, an OTA standard was previously methylated, with a characteristic peak at RT 17.2 min (Figure 17). Figures 14 and 15 shows the chromatograms obtained before and after methylation. OTA was previously identified in sample chromatogram by comparison with one of a standard solution (2 ng/mL) (Figure 12 and 13), submitted under the same conditions. The disappearance of OTA peak is not clear and the appearance of the new peak was difficult to perceive at longer retention time.

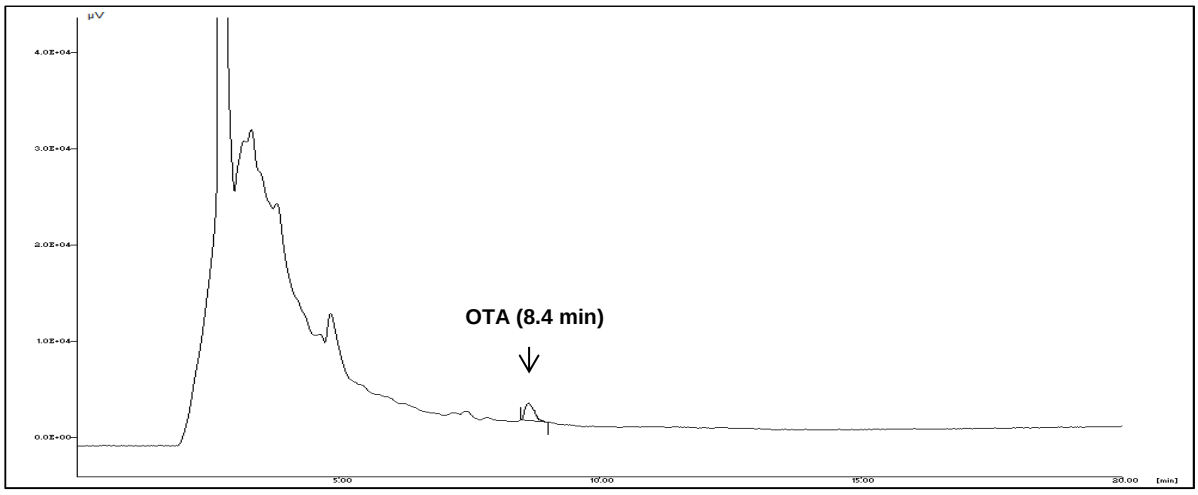


Figure 15.Chromatogram of a coffee substitute sample, before methylation.

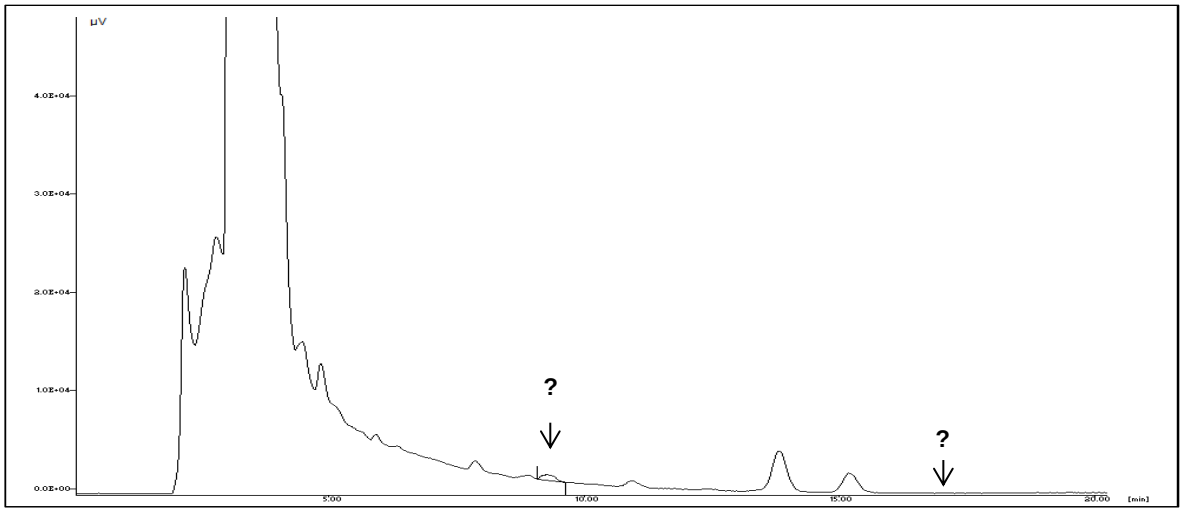


Figure 16. Chromatogram of a coffee substitute sample, after methylation.

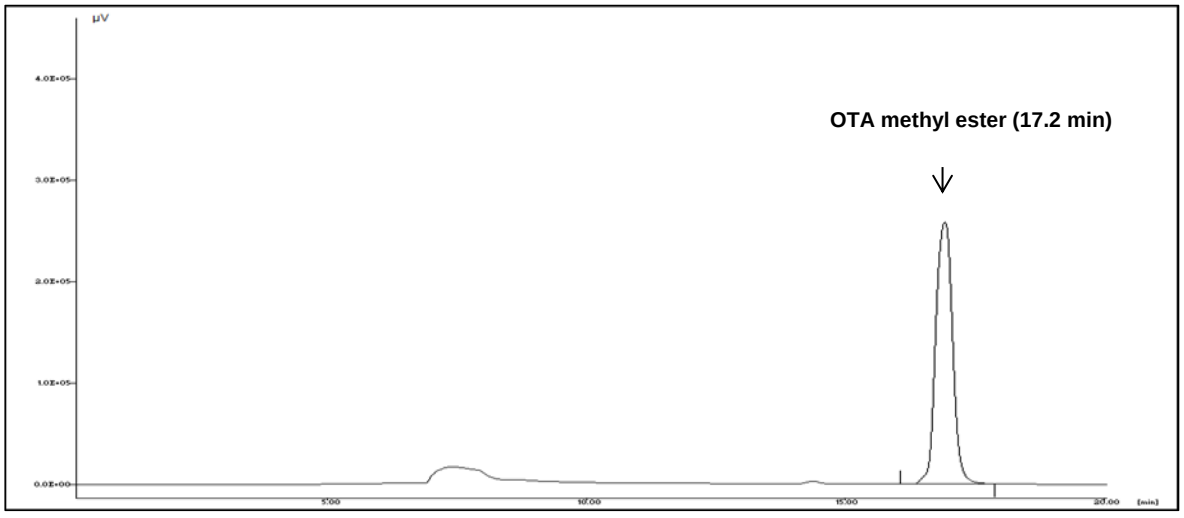


Figure 17.Chromatogram of an OTA standard methylated.

Therefore, a second confirmation attempt was performed, by using a mass spectrometric detector, enabling the identification of the peak on the basis of its molecular fragmentation pattern. As this technique is only available coupled to gas chromatography in the lab where this analytical work was performed, a GC-MS technique was implemented, on the basis of the methodology described by Soleas *et al*. (2001)[98]. For the purpose, the two selected positive samples were extracted by the analytical methodology described previously, and the dry residue was silylated with BSFTA, as described in the material and method section. An OTA standard solution was also subjected to the same procedure.

Figure 18 and 19 represent the derivatized OTA peak on both samples (RT 16.76 min), while Figure 20 details their fragmentation spectra with the characteristic OTA peaks, as described by Soleas *et al*. (2001)[98]. Selective ion monitoring was performed at ions 528, 529, 530, 531, 532, 604, 606 and 619. Based on these observations OTA presence in the samples was confirmed.

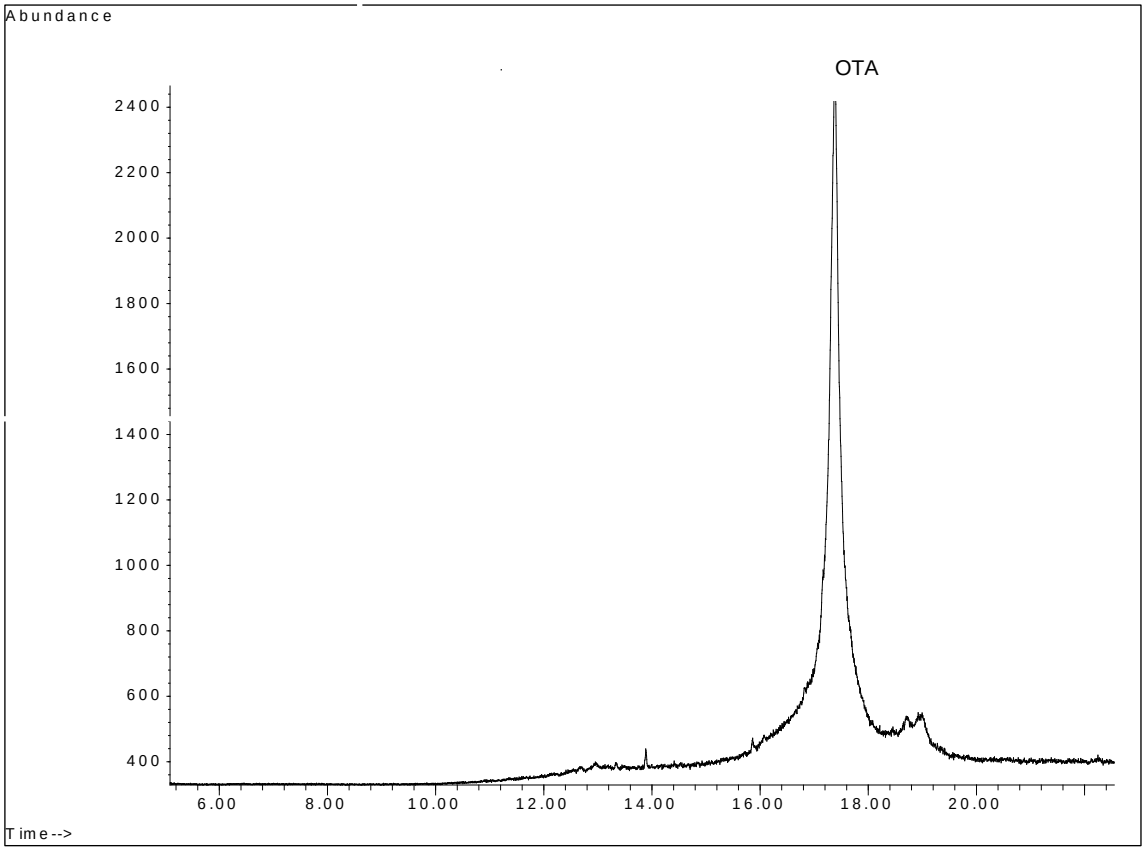


Figure 18. GC chromatogram of a coffee substitute sample.

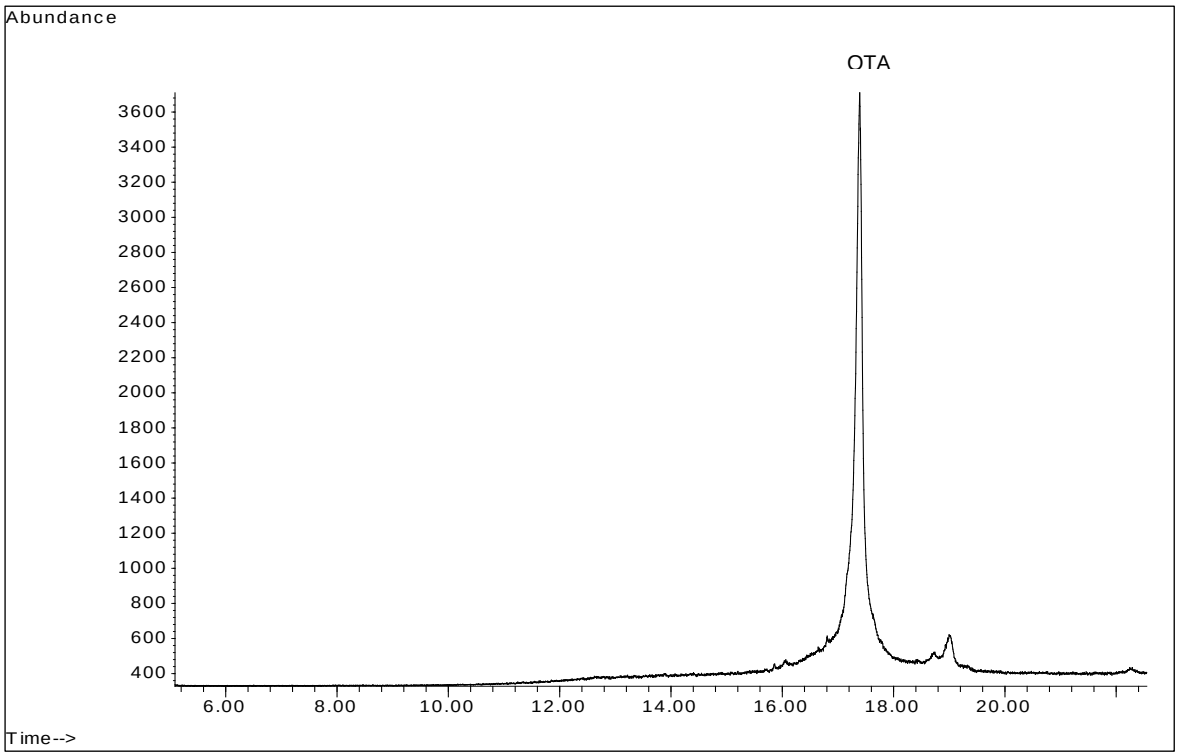


Figure 19. GC chromatogram from a soluble coffee sample.

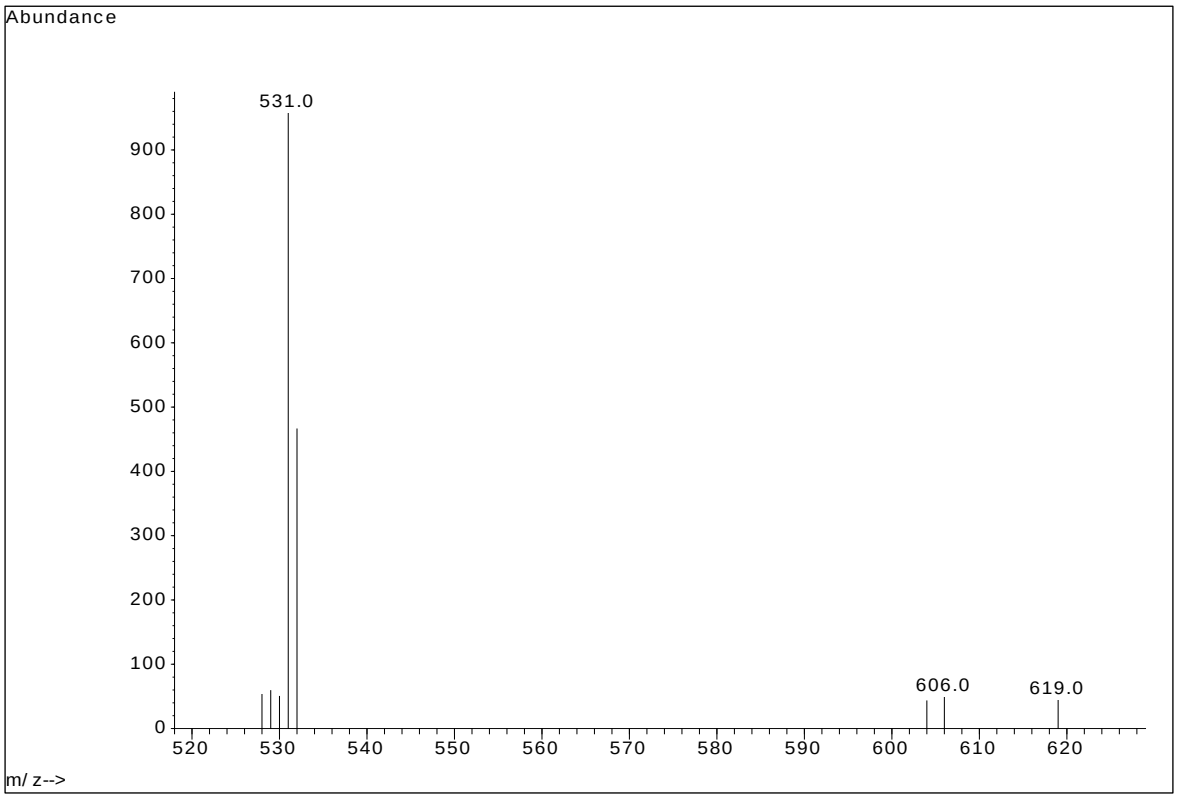


Figure 20. Ion spectrum of OTA (BSTFA derivate).

5.4. Ochratoxins A residues in soluble coffee and coffee substitutes

OTA amounts in soluble coffee and surrogate samples were quantified by the external standard method, as described in the Material and Method section. The methodology was previously validated. As justified, the use of an internal standard would have been the best approach but the diverse attempts proved unsuccessful. Based on the extracted standards calibration curve, the chromatographic response of the sample extracts, injected in triplicate, was converted to ng/mL. The sample amount, extractive solvent volume used in the sample dissolution step, the fraction taken to the immunoaffinity column and the final reconstitution volume of the sample extract, were taken into account for calculating the final results, expressed on a mass basis, as µg/kg.

5.4.1. General overview

The mean results achieved for each sample are detailed in Tables 21 and 22, grouped in 4 categories, as define in the sampling section: soluble coffee; soluble coffee with coffee substitutes, plain substitutes mixtures and barley.

All samples except 7 presented OTA residues above the LOD, ranging from 0.05 to 5.76 µg/kg, highlighting for a consistent presence of this toxin in 83% of the samples analysed. The values, however, were highly variable, even within the groups defined. The highest amount was detected in a soluble coffee (#107 – 5.76 µg/kg) followed by a coffee substitute with added coffee (38%) (#148 – 3.19 µg/kg), both unbranded samples.

The plain soluble coffee groups ranged from 0.06 to 5.76 µg/kg (Table 21). The values achieved and the variability observed are both in agreement with the few results described in the literature. In particular, Vechio *et al*. (2012)[79] quantified 48 samples of soluble coffee collected in supermarkets and retail stores of Italy, reporting values from 0.32 to 6.40 µg/kg while Almeida *et al*. (2012)[78] found 0.17 to 6.29 µg/kg of OTA in the 82 samples purchased from supermarkets in the city of São Paulo (Brazil).

For coffee substitutes, however, literature data are even scarcer. Only one study was found, again from Vechio *et al*. (2012)[79], with values of 0.33-0.52 µg/kg for mixtures of coffee with barley and around 0.51 µg/kg from mixtures of chicory, with 30 to 60% of soluble coffee. These values are apparently lower than the ones described by the same author for soluble coffee. When our values are compared, the plain coffee group also

seems to present higher OTA amounts, an observation that will be discussed later with statistical support.

When compared with the limits established by the European legislation (Table 12) of 10 µg/kg for soluble coffee, all plain soluble samples were clearly below this limit, which can be regarded as an interesting indicative of the quality of the commercialized soluble coffees. As to the substitutes with partial amounts of soluble coffee, if included under the same category, all are also below the 10 µg/kg limit, and apparently below the mean levels quantified for plain soluble coffee.

The coffee substitutes without coffee, prepared from plain barley or a mixture of several roasted cereal extracts are more difficult to classify. If regarded as processed cereals, the European limit is 3 µg/kg (Table 12). If a concentration factor is applied similarly to the one verified with soluble coffee, from 5 µg/kg in the case of roasted and ground coffee to 10 µg/kg in soluble ones, one could estimate a limit around 6 µg/kg for cereals extracts. Since no particular class has been defined, these extracts should be discussed on the basis of processed cereals only. The analysed samples in the present study were all below 0.6 µg/kg, below the established limit. In a recent review, Duarte *et al* (2010) [101] reported that the contamination range for raw barley varied from undetectable to 940 µg/kg, with a wide variation between countries, or even within the same country. These values are comparatively higher than those reported here for roasted barley extracts, which might be a strong indication that the technological process used to prepare the cereal extracts, particularly roasting, can be regarded as a mitigation strategy. Equivalent reductions are described for breakfast cereal processing by extrusion, but not for bread making, highlighting for the importance of the temperature used in the process [101].

Chicory based soluble extracts are even more difficult to discuss under the legal limits, simply because chicory roots are not cereals. As we stated in the theoretical part, a lack of legislation exists to protect from OTA contamination in chicory.

One cannot forget that the industrial method used to prepare these extracts is based on aqueous extraction of roasted raw-materials and that OTA is freely soluble in the hot water used during this process. Therefore, the industrial process potentially concentrates OTA residues. On the other hand, roasting of the raw materials is responsible only for a partial reduction in OTA residues by thermal stress. The main factor, however, is the contamination level of the raw materials used, either green coffee or cereals. The quality of the raw-materials acquired and the storage condition applied are

the main determinants for the OTA levels in soluble coffee and coffee substitutes. Also, despite the conformity of all samples analysed, and the inexistence of a specific class for inclusion of the coffee substitute beverages, one cannot disregard the fact that 83% of the samples presented measurable amounts of OTA, which is indicative of its high prevalence among soluble coffee and its substitutes.

Table 21. Ochratoxin A levels in soluble coffee and coffee substitutes samples with coffee.

Type sample	Sample	Brand	μ [(a)] (μg OTA /kg)	±	SD	RSD (%)
Soluble Coffee	143	C*	0.53	±	0.01	3%
	109	B*	0.56	±	0.04	7%
	108	E*	2.89	±	0.04	1%
	107	D	5.76	±	0.06	1%
	105	F*	0.51	±	0.02	4%
	103	A	0.13	±	0.00	2%
	106	A	0.06	±	0.00	2%
	104	A	0.07	±	0.01	7%
	101	B*	0.71	±	0.03	4%
	102	A	0.99	±	0.03	3%
Cereals with coffee	140	C*	0.56	±	0.04	7%
	138	K*	0.36	±	0.00	1%
	118	J	0.39	±	0.02	6%
	116	F*	0.79	±	0.02	2%
	115	I	2.57	±	0.00	0%
	120	H	0.22	±	0.01	4%
	119	H	0.08	±	0.00	1%
	148	H	3.19	±	0.02	1%
	147	H	0.37	±	0.01	2%
	132	H	1.71	±	0.04	2%
	117	I	0.51	±	0.01	3%
	114	E*	0.23	±	0.00	1%
	149	G	0.05	±	0.00	8%

[a] Each value represents the mean of triplicate injections; * - unbranded.

Table 22. Ochratoxin A levels in coffee substitutes samples without coffee.

Type sample	Sample	Brand	μ [(a)] (μg OTA /kg)	±	SD	RSD (%)
Cereals mixed	139	C*	0.19	±	0.00	3%
	137	K*	<LOD			
	126	H	<LOD			
	122	F*	0.34	±	0.01	2%
	129	D	0.21	±	0.01	5%
	130	N*	<LOD			
	134	L*	0.12	±	0.01	11%
	127	I	0.46	±	0.01	3%
	146	M	<LOD			
	124	H	0.39	±	0.00	0%
	123	H	<LOD			
Barley	136	K*	0.13	±	0.00	2%
	135	L*	<LOD			
	128	I	<LOD			
	125	H	0.61	±	0.00	0%
	121	F*	0.52	±	0.00	0%
Chicory	131	O	0.12	±	0.01	10%

[a] Each value represents the mean of triplicate injections; * - unbranded.

5.4.2. Contribution of the sample type for OTA amounts

In order to understand OTA variability, the results were analyzed statistically for the type of sample, as well as the amount of the constituents, when declared. As a first step, the normality of the dependent variable (OTA µg/kg) in the sample groups was tested by the Shapiro-Wilk test (n<50). Invariably the p-value was inferior to 0.05, indicative that the data do not present a normal distribution, taking in account mainly the reduced number of analytical results. Therefore, in order to evaluate if the sample type affect significantly OTA amounts, a Kruskal-Wallis test was applied, followed by the Mann Whitney Test which compare mean differences between groups sample. The statistical results presuppose the elimination of outliers.

Table 23 describe the results obtained for the coffee or non-coffee groups after eliminating those outliers. Unfortunately, as only two samples were more heavily contaminated, they were eliminated in this process and therefore the results discussion should be interpreted with caution. Without outliers, it is clear that the samples containing coffee, from 100% to 20%, have significantly higher amounts of OTA than the group without coffee (p=0.007).

Table 23. Mean levels of OTA occurrence in samples analysed, with and without coffee.

Type sample	n	µ ± SD (µg OTA/kg sample)
Containing coffee	23	1.01 ± 1.38
Without coffee	13	0.24 ± 0.20

n – number of samples

Table 24 defines the division into the four groups previously defined, which are significantly different (p=0.049). Under this division both groups with coffee (plain coffee and coffee with cereals) are similar, while a subdivision in the non-coffee group between cereal mixtures and plain cereal (barley) is possible but has reduced significance. Again the major differences are determined by the groups defined in Table 23, and therefore coffee presence seems to be the main determinant for the increased OTA amounts.

Table 24. Mean levels of OTA occurrence in selected type samples.

Group sample	n	μ ± SD (μg OTA/kg sample)
Coffee	10	1.22 ± 1.79
Cereals with coffee	13	0.85 ± 1.00
Cereals mixed	8	0.22 ± 0.16
Barley	4	0.32 ± 0.29

n – number of samples

Based on the higher amounts of OTA residues found in the samples containing coffee, one could expect that the coffee amounts could be somehow correlated with OTA content. In order to study this possible association, a 2-tailed Pearson correlation was tested between the labelled coffee amounts and the OTA residues. As observed in Table 25, despite the positive and significant correlation (r = 0.554) at the 0.01 level, the high dispersion of OTA amounts in the 40% coffee group, reduces the significance of this hypothesis. The values for 20% coffee were similar to those presented by the cereals group. When the 100% group was added to this correlation attempt the significance was reduced. These results could be a direct consequence of the quality of the coffees used. When consumers buy plain soluble coffee they expect to obtain a product with a quality as similar as possible to that of fresh ground coffee. Despite being impossible to accomplish, the industries use coffees of higher quality to reduce quality loss during conversion into soluble coffee. In the 40% coffee group one could expect a different strategy: being diluted in a coffee substitute, the amount of coffee is not enough to highlight its characteristics. The presence of coffee retains the physiological effects derived from caffeine, while the beverage is bought at reduced price. Here, the coffee quality is masked by the other components, using probably coffees of comparatively lower quality. Still, one cannot forget that the 40% coffee group is an atypical one in comparison with the others, because it is mixed with chicory only, while in the 20% groups other cereals make the bulk. Therefore, chicory itself could be the main contributor for the increased OTA amounts. Unfortunately only one sample of plain soluble chicory was found in the market and the OTA amounts were low. It would be interesting to study more in detail the contribution of roasted chicory roots to OTA residues, as its high moisture content and direct content with the soil during growing are important factor for potential contamination with fungal species.

Table 25. Relation between OTA levels and coffee amounts in samples analysed.

Coffee (%)	n	μ ± SD (μg OTA/kg sample)
40	4	1.44 ± 1.31
20	9	0.58 ± 0.78
0	13	0.24 ± 0.20

n- number of samples

A similar correlation attempt was also tested for barley and chicory amounts. For the first cereal, samples were grouped from 0 to 100% barley, as detailed in Table 26. The two variables (OTA and barley amounts) are not correlated (p=0.143), according to the weak Pearson correlation (r = 0.195) obtained. Therefore, the increased OTA amounts in some samples are not correlated with barley presence. Indeed, the 100% barley group, a beverage sold frequently for children is in the lower range of OTA residues found in this study.

Table 26. Relation between OTA levels and barley amounts in samples analysed.

Barley (%)	n	μ ± SD (μg OTA/kg sample)
100	4	0.32 ± 0.29
55	4	0.46 ± 0.24
50	3	1.05 ± 1.33
35	3	0.18 ± 0.16
0	7	0.88 ± 1.16

n- number of samples.

For chicory, samples were grouped from 0 to 100% chicory, as detailed in Table 27.

Table 27. Relation between OTA levels and chicory amounts in samples analysed.

Chicory (%)	n	μ ± SD (μg OTA/kg sample)
100	1	0.12 ± 0.12
80	2	0.14 ± 0.95
60	4	0.44 ± 0.07
25	3	0.18 ± 0.14
20	2	0.28 ± 0.18
0[a]	4	0.32 ± 0.29

[a] 100 barley; n- number of samples.

The two variables (OTA and chicory amounts) are also not correlated (p=0.321), according to the weak Pearson correlation (r = -0.191) obtained. Thus, the increased OTA amounts in some samples are not correlated with chicory presence. Indeed, coffee based chicory, a beverage sold for the set of benefits in human health, is also in the lower range of OTA residues found in this study.

5.4.3. The "brand" effect

The analysed samples included for all the classes both branded samples and "unbranded" ones. Knowing that the latter are frequently associated with reduced price, this could indirectly potentiate the used of lower grade raw materials, and therefore influence OTA amounts. Table 28 details the division into branded and unbranded samples within the plain coffee group and the one of substitutes with coffee. In the plain soluble coffee category the OTA amounts were clearly higher in the branded samples, which is in direct contradiction to our previous hypothesis. The same observation can be made in the second, group and even when all samples are treated together. The mean differences between divisions of brand in each group of samples, was compared with Mann Whitney Test, after Kruskal-Wallis means comparison. Because outliers have been previously removed no significant statistical differences were observed (p>0.05).

Table 28. Relation between brand and OTA levels occurrence in samples analysed, containing coffee.

Type sample	Brand	μ ± SD (μg OTA/kg sample)
Coffee (n=10)	Brand	3.07 ± 3.80
	Unbrand	1.14 ± 1.16
Cereals with coffee (n=13)	Brand	0.77 ± 0.68
	Unbrand	0.48 ± 0.24
Average (n=23)	Brand	1.54 ± 2.14
	Unbrand	0.81 ± 0.85

n - number of samples.

Table 29 estimates the same division but for the non-coffee groups, where the results demonstrated previously to be lower than the coffee group. The differences are of reduced significance (p>0.05), and the values are generally low and more homogeneous.

Table 29. Relation between brand and OTA levels occurrence in samples analysed, without coffee.

Type sample	Brand	μ ± SD (μg OTA/kg sample)
One cereal (n=5)	Brand	0.25 ± 0.32
	Unbrand	0.33 ± 0.28
Cereals without coffee (n=7)	Brand	0.29 ± 0.00
	Unbrand	0.17 ± 0.01
Average (n=12)	Brand	0.27 ± 0.00
	Unbrand	0.22 ± 0.00

n - number of samples.

The following table summarizes the mean levels of each brand (Table 30), including within each group all samples from 20 to 100% coffee.

Table 30. Mean levels of OTA occurrence in brands of samples analysed.

Brand	n	μ ± SD (ng OTA/g sample)
B*	2	0.63 ± 0.08 a,c
C*	3	0.41 ± 0.25 b,c
F*	4	0.54 ± 0.18 c
K*	3	0.18 ± 0.16 d
A	4	0.32 ± 0.41 a,b,d
H	8	0.51 ± 0.55 b,c
I	4	0.89 ± 1.03 a,b,c,d
J	1	0.39 ± 0.02 b,c
"Outliers"	7	1.39 ± 2.12 a,b,c,d

* – unbranded; n - number of samples; Different letters in column show statistically differences ($p<0.05$) between means (Kruskal-Wallis Test). Means were compared by Mann-Whitney's Test, since Normal distribution was not confirmed by Shapiro-Wilk's Test ($p<0.05$).

As previously discussed, a high dispersion of results for individual samples was observed, with the higher contaminated samples being considered as outliers from the statistical point of view. Therefore, in the present comparison, brands D, E*, G, L* and O were grouped as "outliers". When the remaining samples are compared, it is possible to

observe higher mean OTA amounts in brand I and lower in brand K, being interesting to refer that the first is a commercial brand while the second is an unbranded group.

Based on these observations, and despite the high variability, the "brand factor" seems not to be a determinant factor for OTA amounts. Indeed, and as previously discussed, coffee and cereals are bought by the industries from several suppliers and, despite having good preservation condition within the industrial facilities, the previous conditions during growing, storage and transportation have a determinant part in these figures.

5.4.4. Estimation of OTA Dietary Intake

As mentioned in the theoretical part, the JECFA and EFSA has set a Provisional Tolerable daily Intake (PTDI) for OTA of 14 and 17 ng/kg bw/day, respectively [50, 51].

Table 31 shows the mean daily intake and contribution of each sample type group analysed to OTA's PTDI. For calculation purposes a daily ingestion of 4 beverages prepared with 2 g of soluble powder (1 tea spoon) was taken into account, which is above the consumption level of most Europeans [70].

Table 31. Mean daily intake and contribution of each sample type group analysed to OTA's PTDI.

Type sample	PTDI* / 60kg bw average	Mean OTA ng/day	Max OTA ng/day	Contribution PTDI (%)	
				Mean OTA	Max OTA
Soluble coffee	1020 ng	9.76	46.08	1.0%	4.5%
Cereals with coffee		6.8	25.52	0.7%	2.5%
Cereals mixed		1.76	3.68	0.2%	0.4%
One Cereal		2.24	4.88	0.2%	0.5%

*EFSA PTWI values correspond to the Provisional Tolerable Daily Intake (PTDI) values of 17 ng/kg bw/day

Plain soluble coffee provides on average 1.0% of PTDI, with an estimated maximum of 4.5%, while for coffee substitutes with coffee the contribution is about a half of that of plain soluble coffee. Cereals contribution is extremely low, achieving an estimated maximum of 0.5% of PTDI.

6. Conclusions

This dissertation intended to provide information on the content of OTA in coffee substitutes consumed regularly by the Portuguese population. It was also a secondary objective to understand the main route of contamination, given that both coffee and cereals are important sources of OTA contamination.

In order to provide greater sensitivity and to minimize the matrix effect of the sample under study, the analytical method was optimized and revalidated, demonstrating to be robust and appropriate for the intended analysis.

As regards to the results obtained, OTA amounts found in samples were highly variable, even within the pre-defined samples groups, highlighting the consistent presence of this toxin in the samples analysed (>80%). Still, OTA amounts were below the limits set by European legislation, despite the inexistence of clear limits for the cereal extracts and chicory. OTA amounts were, on average, significantly higher in the plain soluble coffee group (1.22 µg/kg), followed by coffee with cereals (0.85 µg/kg) and cereals mixtures without coffee (0.22 µg/kg in cereals mix and 0.28 µg/kg in plain cereals). The major differences were determined between coffee and non-coffee groups (1.01 µg/kg *vs* 0.24 µg/kg, respectively) and a significant linear correlation was observed between OTA concentrations and the coffee amount in the mixtures, highlighting that coffee seems to be the main determinant for the OTA amounts. The statistical analysis revealed that the "brand" factor (branded *vs* unbranded) is not determinant for OTA amounts.

Taking into account the preparation instructions recommended by the manufacturers (2 g of powder per beverage) and considering the maximum consumption of four drinks per day, its contribution to OTA ingestion was estimated. The results of this survey indicate that plain soluble coffee and coffee substitutes are not a major source of OTA in the Portuguese diet, providing small amounts, with the estimated intake being well within safety limits.

Being within all safety and legal limits is regarded as a positive conclusion regarding their quality, at least for the samples sold in the Portuguese market, but the high prevalence of positive samples should not be disregarded, particularly from the coffee point of view, being strong indicators of inadequate storage or processing condition in the green coffee chain.

These findings should be a starting point for future studies. Indeed, for more consistent results and sustained conclusions, it would be important:

- Evaluate samples taking into account other technological parameters, as the coffee/cereal geographical origins, storage conditions, roasting degree, and extractive conductions (temperature, time, amount of water).
- Evaluate the contamination with other mycotoxins, particularly aflatoxins, which are highly toxic. Although with scarce information for coffee, their presence in cereals is well documented, and their ingestions is also potential fatal to humans.

7. References

[1] International Coffee Organization (ICO). About Coffee. Available in: www.ico.org/ [accessed in 25/04/2013]

[2] Butt, M. S. and Sultan, M. T. (2011). Coffee and its Consumption: Benefits and Risks. *Critical Reviews in Food Science and Nutrition*, 51(4):363-73.

[3] Casal S. (2004). Compostos nitrogenados do café – Desenvolvimento de metodologias analíticas e sua aplicação na discriminação de espécies e no controlo da intensidade de torra [tese de Doutoramento]. Porto: Faculdade de Farmácia da Universidade do Porto.

[4] Clarke, R. J. (2008). Technology III: Instant Coffee. In: Coffee - Recent Developments (Clarke, R. J. and Vitzthum, O. G., eds), Oxford*: Blackwell Science Limited*, UK.

[5] Oestreich-Janzen, S. (2010). 3.25 - Chemistry of Coffee. In: *Comprehensive Natural Products II*. (Lew, M. and Hung-Wen, L., Eds.), volume 3, p.1085-117. *Elsevier*, Oxford.

[6] Illy, E. (2002).The complexity of Coffee. *Scientific American Magazine*, 286(6):86-91.

[7] Neilson, J. and Pritchard, B. (2009). How to Make a (South Indian) Cup of Tea or Coffee. Value Chain Struggles: Institutions and Governance in the Plantation Districts of South India, *Wiley-Blackwell*, p. 66-106.

[8] Illy, A. and Viani, R. (1995). Espresso Coffee: The Chemistry of Quality. London: *Academic Press Limited*, p. 253.

[9] International Coffee Organization (ICO). Monthly Coffee Market Report - February 2012. Available in: http://www.ico.org/ [accessed in 25/04/2013].

[10] FAOSTAT home page. Production - Crops. Available in http://faostat.fao.org/ [accessed in 12/06/2013]

[11] ChartsBin statistics collector team 2011. Current Worldwide Annual Coffee Consumption per capita 2011. Available in: http://chartsbin.com/view/581 [accessed in 20/08/2013]

[12] Oliveira, M., Casal, S., Morais, S., Alves, C., Dias, F., Ramos. S., *et al*. (2012). Intra- and interspecific mineral composition variability of commercial instant coffees and coffee substitutes: Contribution to mineral intake. *Food Chemistry*, 130(3):702-9.

[13] Macrae, R. and Clarke, R.J (1987). Coffee – Volume 5: Related Beverages, London: *Elsevier Applied Science Publishers*, p. 214.

[14] Geel, L., Kinnear, M. and de Kock, H.L. (2005). Relating consumer preferences to sensory attributes of instant coffee. *Food Quality and Preference*, 16(3):237-44.

[15] Belitz, H-D, Grosch, W. and Schieberle, P. (2009). Coffee and Coffee Substitutes, in Coffee, Tea, Cocoa. *Food Chemistry*: Springer Berlin Heidelberg, p. 938-70.

[16] Radomir, L. (2004). Coffee, Tea and Spices, in Food Quality and Standards. In: Encyclopedia of Life Support Systems (EOLSS): Developed under the Auspices of the UNESCO (Radomir, L., ed.), *EOLSS Publishers*, Oxford ,UK.

[17] Gamito, J. L. C. (2010). A Cultura da Chicória para "Café" (Cichorium intybus L.) na Região do Ribatejo. [tese de Mestrado]: Lisboa: Instituto Superior da Agronomia - Universidade Técnica de Lisboa.

[18] Image of dried, roasted and ground chicory. Available in: http://www.pmkchicory.com/ [accessed in 14/06/2013]

[19] International Coffee Organization (ICO). World trade of soluble coffee. Available in: http://www.ico.org/ [accessed in 25/04/2013].

[20] European Coffee Report 2010/11. The Netherlands: European Coffee Federation. Available in: www.ecf-coffee.org [accessed in 14/06/2013]

[21] International Coffee Organization (ICO). Trends in coffee consumption in selected importing countries. International Coffee Council 109th Session 24 – 28 September 2012 London, United Kingdom, 2012.

[22] Características do Mercado de Cafés Solúveis em Portugal. Revista Cafeicultura. Available in: http://www.revistacafeicultura.com.br/ [accessed in 28/06/2013]

[23] FAOSTAT home page. Trade. Available in: http://faostat.fao.org/ [accessed in12/06/2013]

[24] Food and Agriculture Organization of the United Nations (FAO). Mycotoxins. Available in: http://www.fao.org/ [accessed in 31/10/2012].

[25] Veja, M., Muñoz, K., Sepúlveda, C., Aranda, M., Campos. V., Villegas. R, *et al*. (2009). Solid-phase extraction and HPLC determination of Ochratoxin A in cereals products on Chilean market. *Food Control*, 20(7):631-4.

[26] Bhat, R., Rai, R.V. and Karim, A. A. (2010). Mycotoxins in Food and Feed: Present Status and Future Concerns. *Comprehensive Reviews in Food Science and Food Safety*, 9(1):57-81.

[27] Bennett, J. W. and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews*, 16(3):497-516.

[38] Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses - An overview. *International Journal of Food Microbiology*, 119(1–2):3-10.

[29] Venâncio, A. and Paterson, R. (2007). The Challenge of Mycotoxins. In: Food Safety (McElhatton, A and Marshall, R., eds), USA: *Springer*, p. 26-49.

[30] IARC (1993). Mycotoxins. In IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins, Volume 56, p. 245-521. World Health Organization (WHO), Lyon, France.

[31] Milićević, D.R., Škrinjar, M. and Baltić, T. (2010). Real and Perceived Risks for Mycotoxin Contamination in Foods and Feeds: Challenges for Food Safety Control. *Toxins*, 2(4):572-92.

[32] Richard, J. L., Payne, G. A., Desjardin, A. E., Maragos, C., Norred, W. P., Pestka, J. J., *et al*. (2003). Mycotoxins: risks in plant, animal, and human systems. Ames, Iowa, USA: Council for Agricultural Science and Technology.

[33] Bayman, P. and Baker, J. (2006). Ochratoxins: A global perspective. *Mycopathologia*, 162(3):215-23.

[34] Magan, N., Aldred, D (2005). Conditions of formation of ochratoxin A in drying, transport and in different commodities. *Food Additives and Contaminants*, 22 (sup1):10-6.

[35] Scudamore, K. A. (2005). Prevention of ochratoxin A in commodities and likely effects of processing fractionation and animal feeds. *Food Additives and Contaminants*, 22 (sup1):17-25.

[36] Anli, E., Alkis, İ. M. (2010). Ochratoxin A and Brewing Technology: A Review. *Journal of the Institute of Brewing*, 116(1):23-32.

[37] Amézqueta, S., Schorr-Galindo, S., Murillo-Arbizu, M., González-Peñas, E., López de Cerain, A. and Guiraud, J.P. (2012) OTA-producing fungi in foodstuffs: A review. *Food Control*, 26(2):259-68.

[38] Pohland, A. E., Nesheim and S., Friedman, L. (1992). Ochratoxin A: A review (Thecnical Report). *Pure and Applied Chemistry*, 64(7):1029-46.

[39] El Khoury, A. and Atoui, A. (2010). Ochratoxin A: General Overview and Actual Molecular Status. *Toxins*, 2(4):461-93.

[40] Ribeiro, E. (2007). Contaminação Toxicológica de Resíduos Vitivinícolas – Ocratoxina A [Master Thesis]. Porto: Faculdade de Engenharia da Universidade do Porto.

[41] Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, 15(2):129-44.

[42] O'Brien, E., Heussner, A. H. and Dietrich, D. R. (2001). Species-, sex-, and cell type-specific effects of ochratoxin A and B. *Toxicological sciences: an official journal of the Society of Toxicology*, 63(2):256-64.

[43] Pfohl-Leszkowicz, A and Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition & Food Research*, 51(1):61-99.

[44] Ringot, D., Chango, A., Schneider, Y-J. and Larondelle, Y. (2006) Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chemico-Biological Interactions*, 159(1):18-46.

[45] Breitholtz-Emanuelsson, A., Olsen, M., Oskarsson, A., Palminger, I. and Hult, K. (1993). Ochratoxin A in cow's milk and in human milk with corresponding human blood samples. *Journal of AOAC International*, 76(4):842-6.

[46] Schatzmayr, G., Heidler, D., Fuchs, E., Binder, E., Loibner, A. and Braun, R. (2002). Evidence of ochratoxin A-detoxification activity of rumen fluid, intestinal fluid and soil samples as well as isolation of relevant microorganisms from these environments. *Mycotoxin Research*, 18(0):183-7.

[47] Schaaf, G. J., Nijmeijer, S. M., Maas, R. F. M., Roestenberg, P., de Groene, E. M. and Fink-Gremmels, J. (2002). The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1588(2):149-58.

[48] Schilter, B., Marin-Kuan, M., Delatour, T., Nestler, S., Mantle, P. and Cavin, C. (2005). Ochratoxin A: potential epigenetic mechanisms of toxicity and carcinogenicity. *Food Additives and Contaminants*, 1:88-93.

[49] Pfohl-Leszkowicz, A., Grosse, Y., Kane, A., Creppy, E. E. and Dirheimer, G. (1993). Differential DNA adduct formation and disappearance in three mouse tissues after treatment with the mycotoxin ochratoxin A. *Mutation research*, 289(2):265-73.

[50] JECFA (2007). Joint FAO/WHO Expert Committee on Food Additives (2007). Evaluation of certain food additives and contaminants. In 68th report of the Joint FAO/WHO Expert Committee on food additives. Geneva: WHO.

[51] EFSA (2006). Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Comission related to Ochratoxin A in Food, Question N° EFSA-Q-2005-154, Adopted on 4 April 2006. *The EFSA Journal*, 365(1-56).

[52] Maruya, K., Reddy, V., Wallace, Hayes, A. and Berndt, W. O (1982). Embryocidal, fetotoxic and teratogenic effects of ochratoxin a in rats. *Toxicology*, 25(2–3):175-85.

[53] Hope, H. J. and Hope, B. E. (2012). A Review of the Diagnosis and Treatment of Ochratoxin A Inhalational Exposure Associated with Human Illness and Kidney Disease including Focal Segmental Glomerulosclerosis. *Journal of Environmental and Public Health*, vol. 2012, p. 10

[54] Peraica, M., Radic, B., Lucic, A. and Pavlovic, M (1999). Toxic effects of mycotoxins in humans. *Bull World Health Organ*, 77(9):754-66.

[55] Mycotoxins.info. Animals. Available in: http://www.mycotoxins.info/ [accessed in 22/11/2012]

[56] Van Egmond, H., Schothorst, R. and Jonker, M (2007). Regulations relating to mycotoxins in food. *Analytical and Bioanalytical Chemistry*, 389(1):147-57.

[57] Commission Regulation (EC) No 1881/2006 of 19 December. Setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Communities*. 20/12/2006. L364/5.

[58] Commission Regulation (EC) No 105/2010 of 5 February. Amending Regulation (EC) n° 105/2010 as regards Ochratoxin A toxins. *Official Journal of the European Communitie*s. 6/02/2010. L35/7.

[59] Miraglia, M. and Brera, C (2002). Scientific Cooperation (SCOOP). REPORTS ON TASKS FOR SCIENTIFIC COOPERATION, report of experts participating in Task 3.2.7, Assessment of dietary intake of Ochratoxin A by the population of EU Member States,January 2002. Istituto Superiore di Sanità, Rome, Italy.

[60] Commission Regulation (EC) No 466/2001 of 8 March. Setting maximum levels for certain contaminants in foodstuffs.Official. *Journal of the European Communities*. 16/03/2001. L77/1.

[61] Paulino, De Moraes, M. H. and Luchese, R.H (2003). Ochratoxin A on Green Coffee: Influence of Harvest and Drying Processing Procedures. *Journal of Agricultural and Food Chemistry*, 51(19):5824-8.

[62] Romani, S., Sacchetti, G., Chaves, Lopez, C., Pinnavaia, G. G. and Dalla Rosa, M. (2000). Screening on the Occurrence of Ochratoxin A in Green Coffee Beans of Different Origins and Types. *Journal of Agricultural and Food Chemistry*, 48(8):3616-9.

[63] Studer-Rohr, I., Dietrich, D.R., Schlatter, J. and Schlatter, C. (1995). The occurrence of ochratoxin A in coffee. *Food and Chemical Toxicology*, 33(5):341-55.

[64] Nakajima, M., Tsubouchi, H., Miyabe, M., Ueno, Y. (1997). Survey of aflatoxin B1 and ochratoxin A in commercial green coffee beans by high-performance liquid chromatography linked with immunoaffinity chromatography. *Food and Agricultural Immunology*, 9(2):77-83.

[65] Blanc, M., Pittet, A, Muñoz-Box, R. and Viani, R. (1998). Behavior of Ochratoxin A during Green Coffee Roasting and Soluble Coffee Manufacture. *Journal of Agricultural and Food Chemistry*, 46(2):673-5.

[66] Vanesa, D. and Ana, P. (2013). Occurrence of Ochratoxin A in coffee beans, ground roasted coffee and soluble coffee and method validation. *Food Control*, 30(2):675-8.

[67] Leoni, L. A. B., Valente Soares, L. M. and Oliveira, P. L. C (2000). Ochratoxin A in Brazilian roasted and instant coffees. *Food Additives and Contaminants*, 17(10):867-70.

[68] Prado, G., Oliveira, M. S. D., Abrantes, F. M., Santos, L. G. D., Veloso, T., and Barroso, R. E. D. S. (2000). Incidência de ocratoxina A em café torrado e moído e em café solúvel consumido na cidade de Belo Horizonte, MG. *Food Science and Technology* (Campinas), 20:192-6.

[69] Lombaert, G. A., Pellaers, P., Chettiar, M., Lavalee, D., Scott, P. M. and Lau, B. P.Y. (2002). Survey of Canadian retail coffees for ochratoxin A. *Food Additives and Contaminants*, 19(9):869-77.

[70] Van der Stegen, G., Jörissen, U., Pittet, A., Saccon, M., Steiner, W., Vincenzi, M., *et al*. (1997). Screening of European coffee final products for occurrence of ochratoxin A (OTA). *Food Additives and Contaminants*, 14(3):211-6.

[71] Otteneder, H. and Majerus, P. (2001). Ochratoxin A (OTA) in coffee: nation-wide evaluation of data collected by German Food Control 1995-1999. *Food Additives and Contaminants*, 18(5):431-5.

[72] Coronel, M. B., Marin, S., Cano, G., Ramos, A. J. and Sanchis, V. (2011). Ochratoxin A in Spanish retail ground roasted coffee: Occurrence and assessment of the exposure in Catalonia. *Food Control*, 22(3–4):414-9.

[73] Tsubouchi, H., Yamamoto, K., Hisada, K., Sakabe, Y. and Udagawa, S. (1987). Effect of roasting on ochratoxin A level in green coffee beans inoculated with Aspergillus ochraceus. *Mycopathologia*, 97(2):111-5.

[74] Tabata, S., Lida, K., Kimura, K., Iwasaki, Y., Nakazato, M., Kamata, K., *et al*. (2008). Investigation of ochratoxin A, B and citrinin contamination in various commercial foods. Journal of the Food Hygienic Society of Japan, 49(2):111-5.

[75] Fazekas, B., Tar, A. K. and Zomborszky-Kovács, M (2002). Ochratoxin A contamination of cereal grains and coffee in Hungary in the year 2001. *Acta Veterinaria Hungarica*, 50(2):177-88.

[76] Patel, S., Hazel, C. M., Winterton, A. G. M., Gleadle, A. E. (1997). Survey of ochratoxin A in UK retail coffees. *Food Additives and Contaminants*, 14(3):217-22.

[77] Pittet, A., Tornare, D., Huggett, A. and Viani, R. (1996). Liquid Chromatographic Determination of Ochratoxin A in Pure and Adulterated Soluble Coffee Using an Immunoaffinity Column Cleanup Procedure. *Journal of Agricultural and Food Chemistry*. 44(11):3564-9.

[78] Almeida, A. P. D., Alaburda, J., Shundo, L., Ruvieri, V., Navas, S. A., Lamardo, L. C A., *et al*. (2007). Ochratoxin A in brazilian instant coffee. *Brazilian Journal of Microbiology*, 38:300-3.

[79] Vecchio, A., Mineo, V. and Planeta, D. (2012). Ochratoxin A in instant coffee in Italy. *Food Control*, 28(2):220-3.

[80] Mounjouenpou, P., Justin, F., Guyot, B., Pierre and Guiraud, J. (2012). Study of Ochratoxin: A complexation in coffee. *Asian Journal of Plant Science and Research*, 2(5):570-6.

[81] Tittlemier, S. A., Varga, E., Scott, P. M. and Krska, R. (2011). Sampling of cereals and cereal-based foods for the determination of ochratoxin A: an overview. *Food Additives & Contaminants: Part A*, 28(6):775-85.

[82] Commission Regulation (EC) No 401/2006 of 23 February. as regards laying down methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Communities*. 09/03/2006, L70/12.

[83] Overview of Analytical Methods for Ochratoxin A (Part A: Sample Preparation, Extraction and Clean-Up). Available in: http://www.coffee-ota.org/ [accessed in 05/01/ 2013].

[84] Battalglia, R., Hatzold, T. and Kroes, R. (1996). Conclusions from the Workshop on Ochratoxin in Food, organized by ILSI Europe in Aix-en-Provence (10-12 January 1996). *Food Additives and Contaminants*, 13 Suppl:1-3.

[85] Joint Expert Committee on Food Additives (JECFA): Monographs and Evaluations (JECFA 47 2001). Available in: http://www.inchem.org/pages/jecfa.html [accessed 28/11/2012].

[86] Scott, P. M. (2002). Methods of Analysis for Ochratoxin A. In: Mycotoxins and Food Safety. (DeVries, J. W., Trucksess, M. W. and Jackson, L. S., eds) *Advances in Experimental Medicine and Biology*, volume 504: Springer US. p. 117-34.

[87] Kräutler, O. and Delami, C., G. W. (2000). Proficiency Study Ochratoxin A in Coffee Samples. *Federal Institute for Food Control and Research*. Vienna.

[88] Entwisle, A. C., Williams, A. C., Mann, P. J., Russell, J., Slack, P. T. and Gilbert, J. (2001). Combined phenyl silane and immunoaffinity column cleanup with liquid chromatography for determination of ochratoxin A in roasted coffee: collaborative study. *Journal of AOAC International*, 84(2):444-50.

[89] Vargas, E. A., dos Santos, E.A., Pittet, A., Correa, T.B., da Rocha, A.P., Diaz, G.J., *et al.* (2005). Determination of ochratoxin A in green coffee by immunoaffinity column cleanup and liquid chomatography: collaborative study. *Journal of AOAC International,* 88(3):773-9.

[90] Pittet, A. and Royer, D. (2001). Rapid, Low Cost Thin-Layer Chromatographic Screening Method for the Detection of Ochratoxin A in Green Coffee at a Control Level of 10 µg/kg. *Journal of Agricultural and Food Chemistry*, 50(2):243-7.

[91] Levi, C. P. (1975). Collaborative study of a method for the determination of ochratoxin A in green coffee. *Journal of the Association of Official Analytical Chemists,* 58(2):258-62.

[92] Micco, C., Grossi, M., Miraglia, M. and Brera, C. (1989). A study of the contamination by ochratoxin A of green and roasted coffee beans. *Food Additives and Contaminants*, 6(3):333-9.

[93] Maier, N. M., Buttinger, G., Welhartizki, S., Gavioli, E. and Lindner, W. (2004). Molecularly imprinted polymer-assisted sample clean-up of ochratoxin A from red wine: merits and limitations. *Journal of Chromatography B*, 804(1):103-11.

[94] Rahmani, A., Jinap, S. and Soleimany, F (2009). Qualitative and Quantitative Analysis of Mycotoxins. *Comprehensive Reviews in Food Science and Food Safety*, 8(3):202-51.

[95] Pena, A., Seifrtová, M., Lino, C., Silveira, I. and Solich, P. (2006). Estimation of ochratoxin A in portuguese population: New data on the occurrence in human urine by high performance liquid chromatography with fluorescence detection. *Food and Chemical Toxicology*, 44(9):1449-54.

[96] Zimmerli, B. and Dick, R. (1995). Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. *Journal of Chromatography B: Biomedical Sciences and Applications*, 666(1):85-99.

[97] Association of Official Analytical Chemists (AOAC) (2002). Determination of ochratoxin A in wine and beer. Official Method 2001.01, *AOAC International*.

[98] Soleas, G. J., Yan, J. and Goldberg, D. M. (2001). Assay of ochratoxin A in wine and beer by high-pressure liquid chromatography photodiode array and gas chromatography mass selective detection. *Journal of Agricultural and Food Chemistry*, 49(6):2733-40.

[99] Brito, N. M., De Amarante Junior, O. P., Polese, L. and Ribeiro, M. L. (2003). Validação de Métodos Analíticos: Estratégias e Discussão, *Revista de Ecotoxicologia e Meio Ambiente*, Curitiba, volume 13, 129-146.

[100] Ribani, M., Bottoli, C. B. G., Collins, C. H., Jardim, I. C. S. F. and Melo, L. F. C. (2004). Validação em métodos cromatográficos e eletroforéticos. *Química Nova*, 27:771-80.

[101] Duarte, S. C., Pena, A. and Lino, C. M. (2010). A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. *Food Microbiology*, 27(2):187-98.